



Hypothalamic Nutrient Sensing

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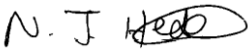
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Declaration

This dissertation is submitted for the degree of Doctor of Philosophy. This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text. No part of this dissertation has been submitted for any other qualification.

Signed: N. J. 

Date: 3rd February 2018

This thesis is dedicated to the memory of my
Grandfather

Cledwyn Thomas

who passed away peacefully on 18th January 2018,
at the age of 92, the day after the final corrections
for my thesis were approved.

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"In his great mercy he has given us new birth into a living hope through the resurrection of Jesus Christ from the dead" 1 Peter Chapter 1 Verse 3 (NIV)

List of Abbreviations

2 – APB - 2-Aminoethoxydiphenyl borate
2 – DG - 2-Deoxy-D-glucose
3V – 3rd ventricle
5 – HT - 5-hydroxytryptamine
AA – amino acid
AAE – amino acid excited
AAI – amino acid inhibited
AAV – adeno – associated virus
aBNST – anterior bed nucleus of the stria terminalis
Act – activated
ACSF – artificial cerebrospinal fluid
AgRP – Agouti Related Peptide
AMP – adenosine monophosphate
AMPK - 5' AMP-activated protein kinase
ANOVA – Analysis Of Variance
APC – anterior pyriform cortex
ARC – Arcuate nucleus
ASD - Autism Spectrum Disorder
ATP – adenosine triphosphate
AUC – area under the curve
Avp – vasopressin
BAT – Brown Adipose Tissue
BCAA – branched chain amino acids
BCAT – Branched chain aminotransferase
BCATm - Branched chain aminotransferase (mitochondrial)
BCKDC - Branched-chain alpha-keto acid dehydrogenase complex
BCKDK - Branched chain ketoacid dehydrogenase kinase
BNST - bed nucleus of the stria terminalis
BSA – Bovine Serum Albumin
CBAL – Core Biochemical Assay Laboratory
CDK - Cyclin-dependent kinase
cDNA - complementary Deoxyribonucleic acid
CFTR - Cystic fibrosis transmembrane conductance regulator
CGRP - Calcitonin gene-related peptide
CIC – alpha chloro-isocaproic acid
CNO - clozapine-N-oxide
CNS – Central Nervous System
CoA – coenzyme A
CRR – Counter regulatory response to hypoglycaemia
CSF – cerebrospinal fluid
DA – dopamine
DEPC - Diethyl pyrocarbonate
DNA - Deoxyribonucleic acid
DREADD – Designer Receptor Activated by Designer Drug
DRP1 - dynamin-related protein 1
EAA – essential amino acid
EGFP – Enhanced Green Fluorescent Protein
eIF2 α – eukaryotic initiation factor 2 α
ER – endoplasmic reticulum or enrichment ratio (please see context)
Erk – Extracellular signal-regulated kinases
FA – fatty acid

FAE – fatty acid excited
 FAI – fatty acid inhibited
 FGF – fibroblast growth factor
 FPKM - Fragments Per Kilobase of transcript per Million mapped reads
 FSIVTGG – frequently sampled intravenous glucose tolerance test
 Gck – glucokinase gene
 GCN2 - general control nonderepressible 2
 GDH – glutamate dehydrogenase
 GDNF - Glial cell-derived neurotrophic factor
 GE – Glucose excited
 GFP - Green Fluorescent Protein
 GI – Glucose inhibited
 GK – Glucokinase
 GKRP – glucokinase regulatory protein
 GLUT – glucose transporter
 GPCR – G Protein Coupled Receptor
 GTP - Guanosine-5'-triphosphate
 GTT – glucose tolerance test
 HAAF – Hypoglycaemia associated autonomic failure
 HFD – high fat diet
 HGP – hepatic glucose production
 HK - hexokinase
 hrGFP - humanized, Renilla reniformis green fluorescent protein
 iBAT - interscapula brown adipose tissue
 i.c.v. – Intracerebroventricular
 IIH – Insulin Induced hypoglycaemia
 IKK2 - inhibitor of nuclear factor kappa-B kinase
 Inh - inhibited
 IP₃ - Inositol trisphosphate
 IP₃R - Inositol trisphosphate receptor
 i.p. – Intraperitoneal injection
 IR – insulin receptor
 IRES - internal ribosome entry site
 ITT – insulin tolerance test
 IVC – Individually Ventilated Cages
 IVGTT – intravenous glucose tolerance test
 KIC - alpha-ketoisocaproate
 KO – knock out
 LAT1 - large neutral amino acid transporter 1
 LepR – Leptin Receptor
 Leu - leucine
 LH – lateral hypothalamus
 MAN – medial amygdala nucleus
 MAP2 - Microtubule-associated protein 2
 MAPK - Mitogen-activated protein kinases
 MBH – Mediobasal Hypothalamus
 MCT - Monocarboxylate transporter
 mGluR – metabotropic glutamate receptor 1
 mRNA – messenger ribonucleic acid
 MSUD - Maple Syrup Urine Disorder
 mTOR – Mammalian Target of Rapamycin
 mTORC1 – Mammalian Target of Rapamycin Complex 1
 NeuN – Neuronal nuclei (Hexaribonucleotide Binding Protein-3)

NG – non glucose sensing
 NGF – nerve growth factor
 nNOS – neuronal nitric oxide synthase
 NO – nitric oxide
 NOS – nitric oxide synthase
 NR – no response
 NPY – Neuropeptide Y
 NTS - Nucleus Tractus Solitarius
 OA – oleic acid
 OAE – oleic acid excited
 OAI – oleic acid inhibited
 OGTT – oral glucose tolerance test
 pS6 – phosphorylated ribosomal protein S6
 PBN – parabrachial nucleus
 PBS – phosphate buffered saline
 PCR – polymerase chain reaction
 PDGF - Platelet-derived growth factor
 PI3K - Phosphatidylinositol-4,5-bisphosphate 3-kinase
 POMC – Pro – Opio Melanocortin
 pS6K1 – Phosphorylated Ribosomal protein S6 kinase 1
 PTT – pyruvate tolerance test
 PVH – paraventricular nucleus of the hypothalamus
 RhoGDI - Rho GDP-dissociation inhibitor
 RNA - ribonucleic acid
 ROS – reactive oxygen species
 RyR – ryanodine receptor
 S6K1 – Ribosomal protein S6 kinase 1
 sGC – soluble guanylyl cyclase
 SGLT – sodium coupled glucose transporter
 SEM – standard error of the mean
 SF1 - Steroidogenic factor 1
 Sst – somatostatin
 Tas1r - Taste receptor type 1 member
 TD-NMR - Time-Domain Nuclear Magnetic Resonance
 Temp - temperature
 TGF - Transforming growth factor
 TH – tyrosine hydroxylase
 TNFR1 - Tumor necrosis factor receptor 1
 TRAP - Translating Ribosome Affinity Purification
 tRNA – transfer ribonucleic acid
 UCP2 – uncoupling protein 2
 VEGF - vascular endothelial growth factor A
 Veh – Vehicle
 VGCC – voltage gated calcium channel
 VMN – Ventromedial Nucleus of the hypothalamus
 WT – wildtype

Summary

Nutrient sensing neurons are unique in coupling changes in the concentration of nutrients to changes in neuronal activity. These neurons typically exist in regions of the brain where the blood brain barrier is fenestrated, such as the arcuate nucleus of the hypothalamus. Glucose and leucine are nutrients known to be sensed by neurons in this brain region, but the mechanisms by which they are sensed, and cells that sense them require further study.

Using calcium imaging of adult neuron cultures from the mouse mediobasal hypothalamus, I demonstrated that leucine bidirectionally regulates neuronal activity in a neurochemically heterogeneous population of neurons, including AgRP/NPY and POMC neurons. Using pharmacological tools, I demonstrated, unexpectedly, that this acute sensing is independent of mTOR and leucine metabolism, known pathways involved in leucine sensing *in vivo*.

Leucine sensing is LAT1 independent. The response principally relies on calcium entry into the cell across the plasma membrane, but IP₃ sensitive calcium stores play a role in neurons inhibited by leucine. Using phosphoTRAP and single cell RNA sequencing, I aimed to identify a molecular marker for leucine sensing cells to allow their manipulation *in vivo*.

PhosphoTRAP, and subsequent pharmacological studies identified a T Type calcium channel may be a marker for leucine sensing cells.

AgRP neurons are essential for feeding, and also play roles in controlling glucose homeostasis. Using chemogenetics to selectively activate these neurons, I demonstrated, in contrast to a similar, recently published study, that blood glucose concentrations did not rise upon activation of these neurons. A subpopulation of AgRP neurons express glucokinase, and some AgRP neurons are glucose inhibited, but the role of glucokinase in these neurons has not been characterised. Our lab generated an AgRP neuron specific glucokinase knock out mouse line. Preliminary results suggest 18 – 25 week old female AgRP glucokinase knock out mice may have altered glucose tolerance, but conclusions can only be drawn once further mice have been phenotyped, and the success of the glucokinase knock out from AgRP neurons has been confirmed.

1 Chapter 1: Introduction

1.1 The necessity of nutrient sensing to life

Nutrients are essential for life. Some nutrients provide energy, others are the basic monomers that form large macromolecules in cells, required for forming cell membranes and the cellular machinery necessary for life (Chantranupong et al., 2015). Ensuring a sufficient supply of these nutrients is essential to survive, and to reproduce to allow life to continue into future generations (Simpson et al., 2015; Solon-Biet et al., 2015).

Nutrient sensing mechanisms exist in organisms, from singled cell bacteria up to humans (Manière et al., 2016; Michel et al., 2007; Oomura et al., 1969; Wu et al., 2006). What constitutes a nutrient varies between organisms; plants, for instance, do not sense amino acids as mammals do, as they synthesise them (Hildebrandt et al., 2015; Singh and Shaner, 1995). Instead, plants sense nitrogen, which is an essential component required to synthesise amino acids, and subsequently to make proteins required for cellular function (Lam et al., 1996).

Nutrients, across evolution, have typically been scarce in the environment, (Chantranupong et al., 2015; Irwin et al., 2015; Milton, 2000), so mechanisms have evolved to sense low, high or a range of nutrient concentrations (Dong et al., 2000; Mesibov et al., 1973; Sourjik and Berg, 2004; Wolfson et al., 2016). Both intracellular and extracellular nutrient sensing mechanisms exist (Dong et al., 2000; Nelson et al., 2002; Sourjik and Berg, 2004). In simple organisms, intracellular sensing mechanisms can sense when cellular nutrient concentrations are low so that organisms know they need to seek out a source of nutrients. In motile bacteria, this involves moving the flagella differently to attempt to move to an area where nutrient concentrations are higher (Berg and Brown, 1972; Ferenci, 2007; Hibbing et al., 2010). In larger, multicellular organisms, coordination between cells or organ systems is necessary to determine whether nutrients are required, and to coordinate responses upon nutrient uptake (Bahn et al., 2007; Bloemendal and Kück, 2013; Morton et al., 2006).

Nutrient storage organs or tissues such as adipose tissue, that have evolved in higher organisms, must sense stored nutrients and communicate with organs that control nutrient seeking behaviour (behaviours such as foraging) (Cordain et al., 2000; Dietrich et al., 2015; Milton, 2000). In static species such as plants, nutrient seeking involves growth towards the

source of nutrients and/or altering the ability to absorb nutrients from the environment in root systems (Chapin, 1980).

In simple unicellular organisms, nutrient sensing mechanisms are necessary when seeking nutrients, to determine if a source of nutrients has been found or not (Chantranupong et al., 2015; Ferenci, 2007; Hibbing et al., 2010; Mesibov et al., 1973; Sourjik and Berg, 2004). In complex, multicellular organisms, intracellular and extracellular nutrient sensing mechanisms in a range of tissues and organs work in coordination to maintain a stable internal environment (Efeyan et al., 2015). “Outward facing” organs such as in the gustatory and digestive systems, possess nutrient sensing mechanisms that are able to inform the body of incoming nutrients (Psichas et al., 2015; Roper and Chaudhari, 2017). It could be argued that nutrient seeking in higher level organisms also involves multiple sensory systems (visual, olfactory, auditory and somatosensory) (Spence et al., 2016). These sensory systems confer information about the environment to aid nutrient seeking. Some have proposed that the evolution of trichromatic vision was driven by a need to forage for fruits (seek nutrients) amongst foliage (Bompas et al., 2013; Regan et al., 2001).

In addition to nutrient seeking behaviour, nutrient sensing controls shifts between anabolic and catabolic processes depending on nutrient abundance (Efeyan et al., 2015). This occurs at the level of the single cell (e.g.: regulating autophagy in eukaryotic cells (Starokadomskyy and Dmytruk, 2013)) and at the whole organism level (e.g.: controlling liver processes such as glycogen synthesis or breakdown (Nordlie et al., 1999)). Controlling these processes is important for survival. High concentrations of some nutrients are toxic (such as ammonium for mammals (Biver et al., 2008)), so possessing sensory mechanisms to detect nutrient excess internally or in the environment are necessary (Chantranupong et al., 2015).

In summary, the necessity of nutrients for life means organisms have evolved sensory mechanisms, to: 1) monitor internal state, to determine nutrient requirements, 2) monitor environmental nutrient abundance, to ensure nutrients can be sourced if required, and 3) regulate the balance between anabolic and catabolic processes.

1.2 Sites of nutrient sensing in the mammalian body

It could be argued that nearly all cells are nutrient sensing, meaning all tissues and organ systems possess the ability to sense nutrients. All cells must be able to alter cellular functions in response to changes in nutrient concentrations, both internally and in the

immediate extracellular environment. The mTOR (mammalian target of rapamycin, or TOR in yeast), GCN2 (general control nonderepressible 2) AMPK (5' AMP-activated protein kinase) signalling pathways, evolutionarily conserved from yeast (Conrad et al., 2014; González and Hall, 2017), sense intracellular energy status and amino acid abundance, to control fundamental cellular processes such as autophagy, transcription/translation and cell division (Chantranupong et al., 2015; Yuan et al., 2013) (Figure 1-1).

However, in large multicellular organisms, additional mechanisms are required to coordinate nutrient handling between multiple organs and tissues, each with different functions and nutrient requirements. Thus, in large multicellular organisms, some cells possess the ability to couple changes in intracellular and/or extracellular nutrient concentrations to changes in electrical activity and/or changes in production of hormones or neurotransmitters, which have effects beyond the sensing cell itself, and possibly beyond the organ system that the cell is a part of. These nutrient concentration changes are sensed by the classical nutrient sensing pathways found in all cells, or by unique nutrient sensing mechanisms (Benford et al., 2017; Efeyan et al., 2015; Hao et al., 2005; Nelson et al., 2002; Wolfson et al., 2016). This ability to sense nutrients and influence other cells, as outlined in Figure 1-1, in the brain, will be the focus of my thesis.

Multiple tissues in higher organisms have cells that possess this ability, which are outlined in Figure 1-2. They are colour coded based on the tissue or organ's ability to sense protein, glucose and/or fatty acids. Different sites are specialised for sensing different nutritional states, and serve different purposes, but they are all positioned within organs or tissues where sensing nutrients provides information about nutrients available for the whole body (not just in the immediate extracellular environment that would likely only be available for that cell or organ) (Kosse et al., 2015; Miyamoto et al., 2013).

These sensing cells typically acquire this information due to their positioning at key sites within the body, where they can sense incoming nutrients (Efeyan et al., 2015; Miyamoto et al., 2013). They then send information to the rest of the body about the systemic availability of nutrients, or nutrients that are expected to enter the circulation (Chen et al., 2015; Morton et al., 2006; Myers and Olson, 2012; Psichas et al., 2015). These mechanisms can act to prime the body for nutrient ingestion, shifting the body's metabolic state from fasted to fed (Berg et al., 2002). For instance, the gustatory and digestive system sense incoming

nutrients before entry into the bloodstream (Gribble and Reimann, 2016; Psichas et al., 2015). Other nutrient sensing sites are found in the portal vein, where glucose principally (and other nutrients indirectly (Mithieux, 2014)) is sensed having exited the GI tract, but before entering the liver. In circumventricular organs of the brain, incoming nutrients to the brain are sensed (Benford et al., 2017; Elmquist et al., 1999; Haddad-Tóvolli et al., 2017).

Long – term, high concentrations of nutrients in the body can be dangerous. As is seen in diabetes, when brain and peripheral glucose sensing becomes defective (Schwartz et al., 2013; Song and Routh, 2006), hyperglycaemia can lead to a range of symptoms, including neuropathy, nephropathy, and eye problems (Chen et al., 2012; Ogunnowo-Bada et al., 2014). Imbalanced brain amino acid uptake, resulting in high concentrations of some amino acids in the brain, disrupts normal brain function (Tărlungeanu et al., 2016; Yudkoff et al., 2005). Ensuring proper clearance from the bloodstream, and storage if necessary, of nutrients is important, which is reliant on mechanisms to sense high nutrient concentrations (Joly-Amado et al., 2012; MacDonald et al., 2005; Postic et al., 1999; Su et al., 2012).

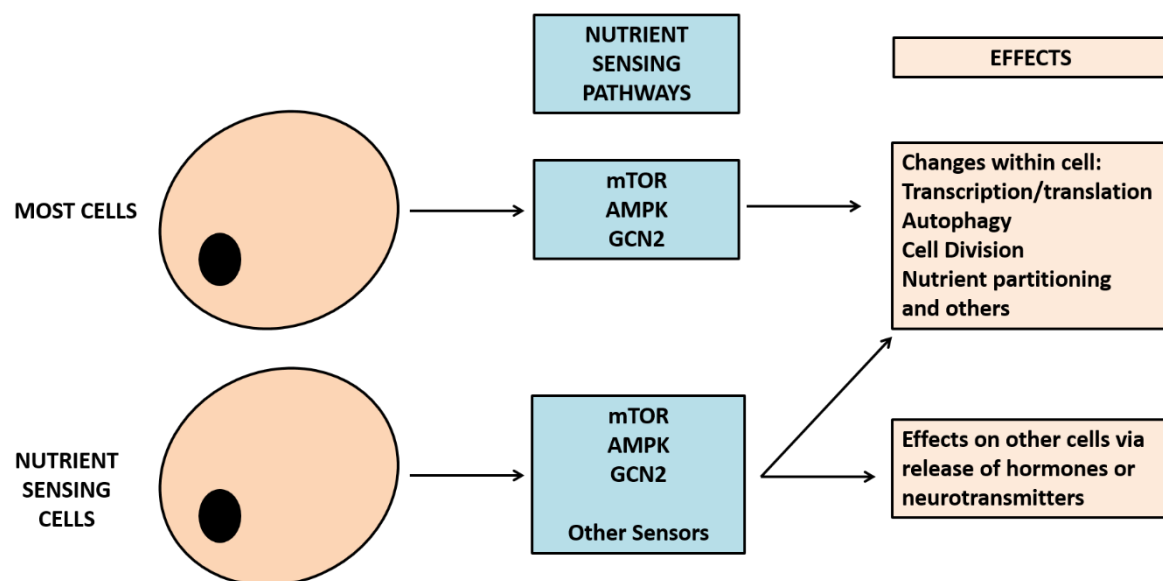


Figure 1-1: Nutrient sensing cells.

Nutrient sensing cells possess the unique ability to couple changes in intracellular or extracellular nutrient concentrations, to changes in the release of chemicals (hormones or neurotransmitters) that affect other cells, within or beyond the same tissue or organ.

Given that there are multiple peripheral nutrient sensors, why does the brain need to sense nutrients directly? Why can the brain not integrate peripheral information, to generate responses? Why has nutrient sensing seen in lower organisms not disappeared in the brain

with evolution, as the periphery relays information to the brain? This is discussed in the next section.

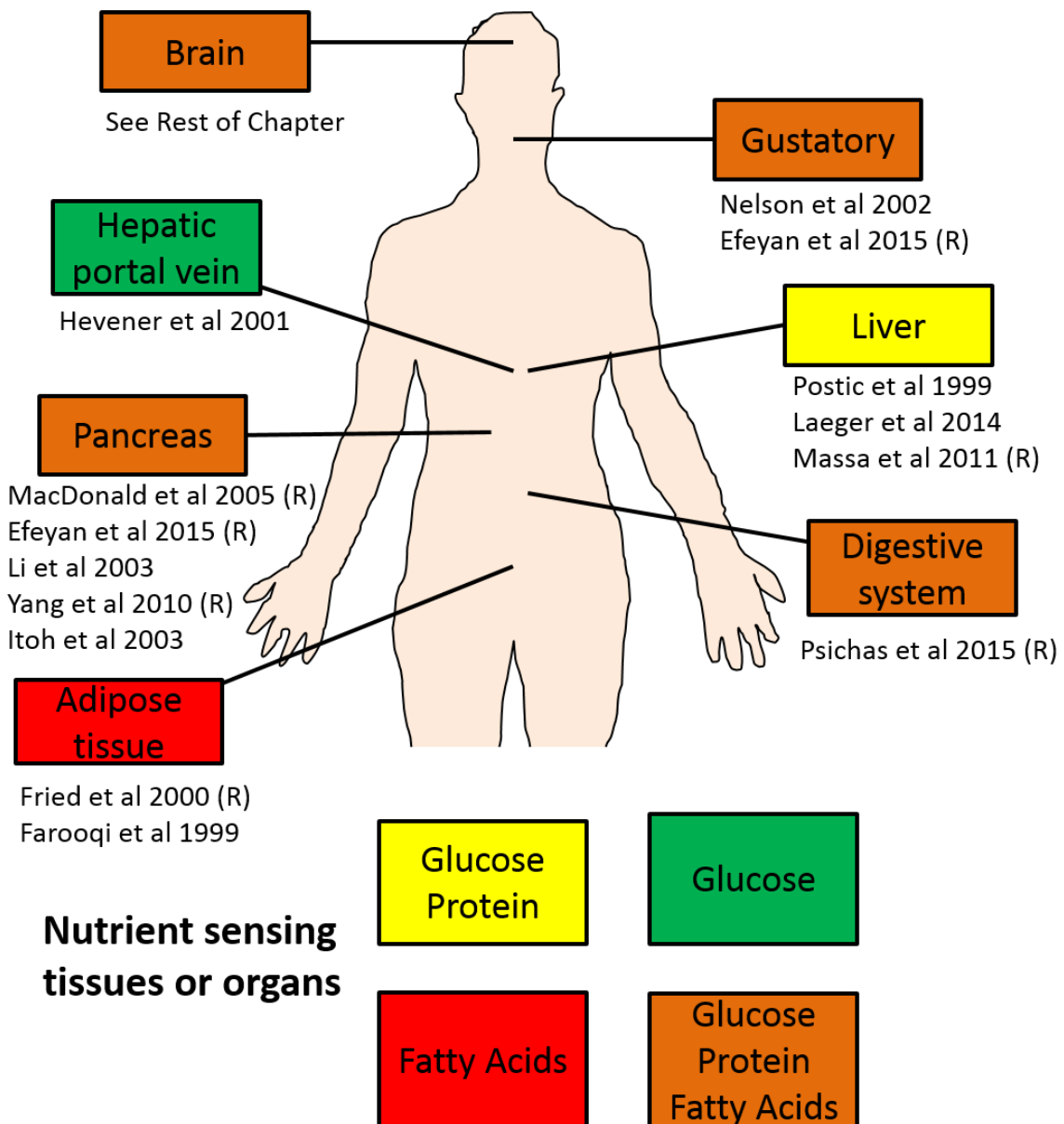


Figure 1-2: Sites of nutrient sensing in the body.

Evidence for each organ or tissues role in nutrient sensing in papers or reviews (R) below each box.

1.3 Why are nutrients sensed in the brain?

Multiple brain regions have been demonstrated to play a role in glucose and amino acid sensing (summarised in these reviews Heeley and Blouet, 2016; Ogunnowo-Bada et al., 2014 and discussed in section 1.4), and the sensing of these nutrients in the brain will be the focus for the rest of my thesis. Most of these brain regions are also sensitive to peripheral signals

of nutrient abundance, such as leptin, ghrelin, and gut hormones (Blouet and Schwartz, 2012; Kohno et al., 2003; Mountjoy et al., 2007; Wang et al., 2014), suggesting they may integrate nutritional information from different sources, discussed shortly.

1.3.1 The selfish brain: the necessity of correct nutrient concentrations for normal brain function

The brain must ensure it has a sufficient supply of nutrients to function correctly, so directly senses the nutrients supplied to it. It can then alter this supply, if needed. Glucose is the brain's primary fuel (Erbsloh et al., 1958; Mergenthaler et al., 2013; Routh et al., 2014). The energy demands of the brain are extremely high, accounting for 20 – 50% of the body's glucose usage depending on age (Erbsloh et al., 1958; Kuzawa et al., 2014; McCrimmon, 2009; Mergenthaler et al., 2013); inadequate glucose supply for the brain, as seen during severe hypoglycaemia during insulin therapy to treat diabetes, can lead to coma or death (Beall et al., 2012; McCrimmon and Sherwin, 2010; Ogunnowo-Bada et al., 2014). Amino acids such as leucine are necessary for neurotransmitter synthesis (Yudkoff et al., 2005), as well as general protein synthesis in the brain, and so an adequate amino acid supply must also be maintained.

If the brain perceives that the concentration of glucose is low, it can act, independently of peripheral signals, to mobilise glucose to the brain. Induction of local hypoglycaemia in the VMN (ventromedial nucleus of hypothalamus) of rats, by injection of a non metabolisable glucose analogue (Borg et al., 1995), caused the initiation of counter regulatory response (CRR) and a rise in blood glucose concentrations. Direct glucose infusion into the VMN of rats during a hypoglycaemic clamp significantly reduced counter regulatory responses (Borg et al., 1997), demonstrating that if the VMN perceives it has enough glucose, it will prevent glucose mobilisation. Optogenetic activation or inhibition of neurons in this brain region mimics these effects (Meek et al., 2016; Stanley et al., 2016). These studies demonstrate VMN glucose sensing is necessary for a full CRR, and sufficient to initiate (at least a partial) CRR. Thus, the brain is able to act “selfishly”, mobilising glucose to itself (Routh et al., 2014). The brain may prioritise its needs over those of other systems, such as the reproductive system, in times of energy deficit, although this hypothesis needs further confirmation (Roland and Moenter, 2011; Routh et al., 2014).

The brain may also be “selfish” for amino acids. Amino acid deprivation in the anterior pyramidal cortex (APC) mimicked by injection of an amino alcohol, generates an aversive response to diets devoid of the corresponding amino acid (Hao et al., 2005), and increases preference for diets containing the corresponding amino acid (Gietzen et al., 1992); rejection of a diet devoid of a specific amino acid is prevented by injection of the limiting amino acid into the APC (Beverly et al., 1990; Russell et al., 2003). It must be noted though that these studies rely on prior reductions in circulating amino acid concentrations through fasting (Gietzen et al., 2016)(discussed in depth in section 1.4.2).

1.3.2 The brain integrates nutritional information, to coordinate nutrient intake and partitioning

While all organs need to ensure adequate nutrient supply, the brain uniquely has the ability to control the functions of nearly all other organ systems and tissues. The brain also controls an animal’s behaviour. The brain receives signals from distributed sites, and controls their function. The brain is the organ that coordinates responses to changes in nutrient concentrations (in the brain, in the circulation, in stores and when nutrients enter the body), based on the integration of nutritional information it receives from distributed sites within the body, or has sensed directly. The brain must have a reliable measure of the organism’s (and the brain’s) internal energy state, to match intake to energy needs over time. Using both hormonal and direct nutrient sensing in combination allows the most accurate response to be initiated. The responses the brain regulates include the initiation of nutrient intake (Luquet et al., 2005), which can include a drive to eat specific nutrients (Gietzen et al., 1992), and the control of nutrient partitioning around the body (Joly-Amado et al., 2012; Morton et al., 2006). This includes shifting metabolic functions in the body from the fasted to the fed state, ensuring nutrients enter tissues where they are required, and that empty nutrient stores (e.g.: liver glycogen) are replenished (Berg et al., 2002; Marty et al., 2007; Ruud et al., 2017).

Nutritional integration has been studied in the hindbrain (Blouet and Schwartz, 2012).

Leucine injection into the NTS (Nucleus of the Solitary Tract) suppresses first meal size after a fast; this suppression is greater when leucine is co injected with doses of leptin or an MC4R (melanocortin 4 receptor) activator that do not by themselves have an anorectic effect (Blouet and Schwartz, 2012), or with i.p. CCK (Cholecystokinin) (Blouet and Schwartz, 2012). The cells that act as integrators may be important in shifting the body from the fasted to fed

state. The anorectic response is only maximal when multiple signals (nutrients, hormones from the gut or nutrient stores, or signals from other brain regions) are present. The brain integrates information to generate a coordinated response. This integrative mechanism is how the brain appears to function, but why can the brain not rely on either peripheral signals or brain nutrient sensing alone?

1.3.3 Why does the brain not rely on peripheral signals alone?

The brain may not rely on peripheral signals alone as many of these, for instance, hormones produced peripherally in response to nutrient abundance, are not specific readouts of the abundance of a single nutrient. Insulin secretion is not just due to high glucose concentrations, but can also be influenced by leucine and fatty acids (Itoh et al., 2003; Li et al., 2003; Yang et al., 2010). Leptin production is influenced not only by fatty acids in adipose tissue, but other factors such as insulin and glucocorticoids (Farooqi et al., 1999; Fried et al., 2000). So, the brain directly senses nutrients to determine the nutrient's abundance in the circulation – nutrients that are immediately available for the brain.

Retaining the ability to sense nutrients is also important so the brain knows exactly how much fuel is reaching it. Peripheral signals are not necessarily an accurate readout of the nutrients reaching the brain (Chen et al., 2015). If, for instance, the brain relied on information from taste alone, although food may have a rewarding taste, it is of no nutritional value if it does not reach the brain (Iordanidou and Burdakov, 2016). Sweet taste receptor knock out mice still choose sugary drinks, suggesting the nutritional and rewarding value of sugar is not sensed by taste alone (de Araujo et al., 2008; Domingos et al., 2013). Recent work suggests that the smell of food can inhibit the activity of food seeking AgRP/NPY (Agouti related Peptide and Neuropeptide Y) neurons in the hypothalamus, but if food is not ingested (i.e.: the signal is wrong) then the activity of these food seeking neurons is restored (Chen and Knight, 2016; Chen et al., 2015).

The brain, as discussed in section 1.3.1, possesses the ability to sense deprivation of specific nutrients. Direct nutrient sensing by the brain allows the brain to integrate the true concentrations of nutrients reaching it with peripheral signals, suggesting that nutrient sensing by the brain may act to “fine – tune” the information received from other sources (Kosse et al., 2015). This is supported by evidence that the glucose sensing neurons of the MBH (mediobasal hypothalamus, which includes, but is not limited to the arcuate and

ventromedial nuclei) are regulated by fasting (Murphy et al., 2009a), and other peripheral signals such as insulin (Cotero and Routh, 2009; Kang et al., 2004; Kohno et al., 2003; Lee et al., 2015; Santoro et al., 2017).

1.3.4 Why does the brain not rely on nutrient sensing alone?

The question (section 1.3.3) can be asked in reverse, why does the brain not rely on nutrient sensing alone? Firstly, circulating nutrients are not an accurate readout of stored nutrients (Hill et al., 2010; Morton and Schwartz, 2011; Morton et al., 2014). If the brain is to make decisions based on the whole body's energy status, it must know the status of energy stores such as adipose tissue and liver glycogen. This may be particularly important in the control of longer term energy requiring processes such as reproduction (Dietrich and Horvath, 2012; Hill et al., 2010; Plum et al., 2005; Routh et al., 2014; Wu et al., 2012).

Secondly, peripheral signals, such as from the gustatory and digestive signals can serve as feed forward signals (Kosse et al., 2015) or gatekeepers, particularly when nutrients are ingested, preparing the body for the arrival of nutrients. This may be a reason why the nutrient sensors found in the body are at sites of initial nutrient contact (e.g.: mouth, gut), and why nutrient sensors in the brain are in circumventricular organs, where the brain tissue is in close contact to the periphery (Levin et al., 2011). This allows the body to respond rapidly when changes in nutrient concentrations occur. Emerging evidence has shown that the olfactory system may play a longer term role in the energy homeostasis (Riera et al., 2017), as may gut microbiota (Le Chatelier et al., 2013; Ridaura et al., 2013), suggesting this view is over simplified.

Another possible explanation for the existence of nutrient sensing in the brain is the time course over which nutrient sensing has evolved (Chantranupong et al., 2015). Nutrient sensing mechanisms existed before the existence of indirect sensing mechanisms. The brain may have retained the ability to sense nutrients, as other sensing pathways have evolved. Other peripheral sensing pathways may exert a greater effect, but the ability to sense nutrients directly is retained (Bruning et al., 2000; Plum et al., 2005; Routh et al., 2014; Schwartz, 2000). Some of the pathways involved in glucose and leucine sensing, mTOR and AMPK, are also controlled by hormones such as insulin and leptin, which could suggest latter regulation added into this system (Cota et al., 2006; Flier, 2006).

In summary, the brain acts as an integrator of peripheral signals, and possesses the ability to sense changes in nutrient concentrations itself. The brain retains the ability to respond to nutrients itself, to ensure it has an accurate measure of the nutrients reaching it (Iordanidou and Burdakov, 2016; Kosse et al., 2015). However, it does make use of peripheral signals, which can control important brain functions (Morton et al., 2006; Varela and Horvath, 2012). Nutrients may play a modulatory role in this context (Canabal et al., 2007a, 2007b; Kang et al., 2004; Kohno et al., 2003), to ensure correct integration of these peripheral signals to control outputs. These two, interlinked reasons may explain why nutrients are sensed directly by the brain. Where in the brain nutrients are sensed, the effects nutrient sensing in the brain control, and the mechanisms by which they are sensed, are discussed in the next three sections.

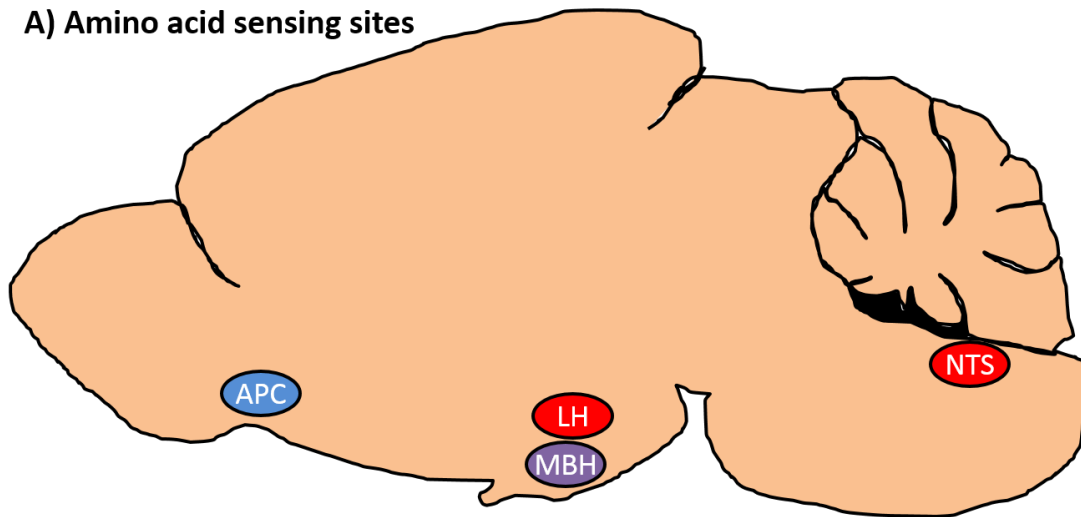
1.4 Brain regions where glucose and amino acids are sensed

1.4.1 Requirements for a nutrient sensing brain region

A brain nutrient sensing region must, a) be exposed to changes in nutrient concentrations and b) possess cells able to sense these changes in nutrient concentrations. All brain regions are exposed to changes in nutrient concentrations, but the time course of changes, and concentration range they are exposed to, will vary.

Brain nutrient sensing sites typically reside in or near to circumventricular regions where the blood brain barrier is leaky (Blouet and Schwartz, 2012; Blouet et al., 2009; Langlet et al., 2013; Oomura et al., 1969; Ritter et al., 1981). This allows them to rapidly respond to changes in nutrient concentrations in the bloodstream, and may mean that cells in these regions are exposed to a wider nutrient concentration range. The median eminence and area postrema are key circumventricular organs in the brain (Levin et al., 2011). Nutrient sensing sites such as the arcuate nucleus (which may at times have a plastic blood brain barrier, see section 1.4.3) and NTS, reside near to these regions. Due to their ability to sense signals or peripheral nutrient abundance (Blouet and Schwartz, 2012; Kohno et al., 2003; Mountjoy et al., 2007; Wang et al., 2014), these sites may act as integrators of nutritional information, allowing a single, coordinated response to be initiated (Morton et al., 2006). In Figure 1-3, primary glucose and amino acid sensing sites in the brain are identified, labelled as to whether they sense low or high concentrations, or both.

A) Amino acid sensing sites



B) Glucose sensing sites

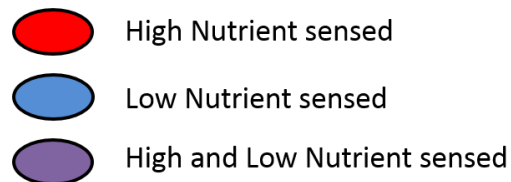
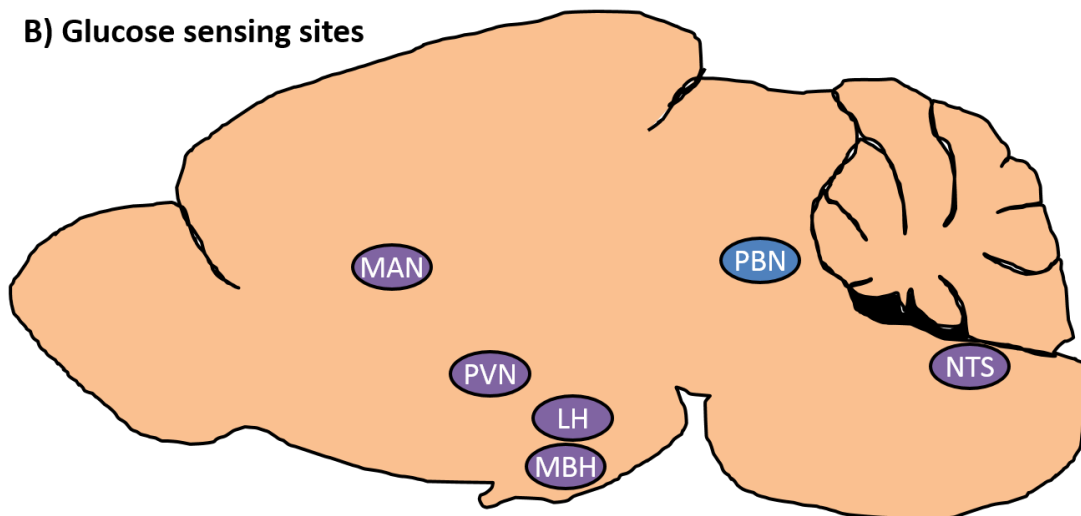


Figure 1-3: Brain regions where nutrients are sensed.

A) Brain regions where amino acids are sensed, B) Brain regions where glucose is sensed. APC – anterior pyriform cortex, LH – lateral hypothalamus, MAN – medial amygdala nucleus, MBH – mediobasal hypothalamus, NTS – Nucleus of the Solitary Tract, PBN – parabrachial nucleus, PVH – paraventricular nucleus of the hypothalamus. Red – sites where high nutrient concentrations are sensed. Blue – sites where low nutrient concentrations are sensed. Purple – sites where both may be sensed. All sensing mechanisms detect changes in nutrient concentrations, but many pathways sense nutrients in specific concentration ranges, allowing increased sensitivity within the range of concentrations that they sense nutrients

1.4.2 Brain regions where amino acids are sensed

1.4.2.1 Brain regions where low amino acid concentrations are sensed

The majority of studies have tested sensing of essential amino acids (amino acids that the body cannot synthesise) in the brain. Diets extremely low in protein (<5%) or devoid of an essential amino acid, which reduce energy intake and growth (Willcock and Hopkins, 1906), can be sensed by the brain. These effects have been shown to be mediated by the anterior piriform cortex, which can sense reductions in essential amino acid concentrations (Gietzen and Rogers, 2006; Gietzen et al., 1986, 2007). Specifically, this brain region mediates the rapid aversive response to diets devoid of one essential amino acid (Beverly et al., 1990; Koehnle et al., 2003, 2004). The APC also has a role in the learned taste aversion to diets devoid of one essential amino acid, and learned preference for diets containing the limiting essential amino acid (Gietzen et al., 1992, 2007). *Ex vivo* studies using electrophysiology have shown neurons from this brain area respond to low amino acid concentrations, or amino alcohols, that mimic low amino acid concentrations by causing tRNA uncharging (Hao et al., 2005; Rudell et al., 2011). Injection of these amino alcohols into the APC stimulates feeding, an effect that is GCN2 dependent (Hao et al., 2005; Maurin et al., 2005).

This large body of work has recently been challenged by the Knight lab, who were not able to replicate the aversive dietary response to diets devoid of amino acids, unless the mice were in a specific nutritional state (Leib and Knight, 2015). The aversive response they did see was GCN2 independent. The inability to reproduce the aversive response is surprising, given that this effect has been reported independently by several labs over many years (e.g.: Hao et al., 2005; Maurin et al., 2005). Additionally, the authors question how this brain region can sense nutrients, as unlike other nutrient sensing regions such as the MBH and NTS, the APC does not have privileged access to the bloodstream or lie near to the ventricular system (Leib and Knight, 2015, 2016). Studies though have demonstrated that concentration changes of amino acids do occur within the plasma, and subsequently within the APC (Feurté et al., 1999; Koehnle et al., 2004; Maurin et al., 2005), within the timeframe of the aversive response, after exposure to diets with the limiting amino acid (just 20 – 40 minutes). This shows that these neurons are responding to changes in nutrient concentrations in the APC, but only when the animals are in a specific nutritional state. Specifically, animals must be fasted or fed a diet devoid of the essential amino acid being

tested for the effect to be observed (Gietzen et al., 2016), which cause low concentrations of plasma amino acids.

The MBH has also been suggested to be a site where this aversive response to diets devoid of a single essential amino acid may be mediated (Maurin et al., 2014). A leucine devoid meal after an overnight fast increased eIF2 α phosphorylation (downstream target of GCN2) in the MBH, and was associated with neuronal activation in the MBH. Adenoviral mediated knockdown of GCN2 in the MBH blunted the anorectic response to a leucine devoid meal, compared to a control diet, after an overnight fast, during an hour of consumption of the diet. I.c.v. L - leucinol injection increased eIF2 α phosphorylation selectively in the MBH but not APC, and inhibited feeding in WT, but not GCN2 KO mice. These data indicate that the MBH is sufficient to initiate an aversive response to a leucine imbalanced diet.

However, although molecular data supports a role for the MBH in sensing low concentrations of amino acids, another study did not observe changes in amino acid concentrations in three hypothalamic nuclei, including the VMH (arcuate and ventromedial nuclei of the hypothalamus) (Gietzen et al., 1989), when animals were fed amino acid imbalanced diets, although these studies were performed with isoleucine and threonine, not leucine. Changes in amino acid concentrations in similar paradigms do occur in the APC (Gietzen et al., 1986; Koehnle et al., 2004). These studies highlight an important point. Changes in the concentration of amino acids in different brain regions will vary. This could, be due to differential expression of amino acid transporters on the blood brain barrier in different brain regions (Betz and Goldstein, 1978; Hawkins et al., 2006; Oldendorf, 1971) (see (Heeley and Blouet, 2016) for further discussion). As many amino acids share the same transporters, competition effects can cause amino acid uptake to vary in different brain regions. Plasma amino acid concentrations will not always directly correlate with brain amino acid concentrations. Thus, some regions may be better positioned to serve as sensing sites for amino acids.

1.4.2.2 Brain regions where high amino acid concentrations are sensed

The MBH and NTS have been demonstrated to be brain regions where increases in essential amino acid concentrations are sensed, specifically changes in concentration of the amino acid leucine. Of all the amino acids, concentrations of the BCAA's (branched chain amino acids, leucine, isoleucine and valine) increase postprandially, while changes in the other

amino acids are smaller or do not increase (Blouet et al., 2009; Glaeser et al., 1983; Voog and Eriksson, 1992), suggesting BCAA's serve as a signal of meal consumption. Of all the essential amino acids that have been tested, leucine is the only one to produce an anorectic effect upon i.c.v. or MBH injection (Blouet et al., 2009; Heeley and Blouet, 2016; Laeger et al., 2014b; Morrison et al., 2007). Studies demonstrating this effect are summarised in Table 1 of Heeley and Blouet, 2016. The anorectic effect is due to changes at multiple levels in the feeding sequence. The signalling pathways so far demonstrated to be responsible for this effect are discussed in sections 1.5.1 and 1.6.1, and in chapter 2.

Non-essential amino acids can also be sensed in the hypothalamus. Orexin/hypocretin neurons, an orexigenic neuronal population in the lateral hypothalamus, have been shown using cFOS immunohistochemistry, after direct hypothalamic infusion, and using slice electrophysiology, to be regulated by non-essential amino acids (Karnani et al., 2011). These neurons were insensitive to changes in essential amino acid concentrations. The depolarization was mediated by System A amino acid transporters and suppression of the hyperpolarizing activity of K_{ATP} channels, but was mTOR independent. The behavioural and metabolic consequences were not examined, but orexin neurons are important mediators of arousal (Sakurai, 2007).

In summary, four sites that sense amino acids in the brain have been identified, with each site projecting to at least one of the others (Blevins et al., 2004; Blouet et al., 2009; Gietzen et al., 1998, 2007), suggesting the existence of an amino acid sensing network in the brain, that may act in coordination to generate outputs in response to changing amino acid concentrations.

1.4.3 Brain regions where glucose is sensed

Early studies demonstrated the existence of glucose sensing cells in the hypothalamus (Oomura et al., 1969), with multiple glucose sensing mechanisms subsequently implicated in the nuclei in this brain region from numerous studies (summarised elegantly in (Routh et al., 2014)). During normal physiology, the principle reason that blood glucose concentrations fall is due to prolonged fasting. The more recent interest in hypoglycaemia detection by the brain, caused by intensive insulin therapy to treat diabetes, is not a physiological phenomenon. This means studies of hypoglycaemia detection must be considered in the context in which they normally occur. A recent study demonstrated glucose concentrations,

as measured by microdialysis, paradoxically increased in the arcuate nucleus after a 24 hour fast, compared to fed mice (Langlet et al., 2013). This was due to glucose induced alterations in the structure of the blood brain barrier, mediated by VEGF (vascular endothelial growth factor A) signalling in hypothalamic tanycytes. However, although arcuate glucose concentrations increased from 1 to 2mM during a fast, they did not reach the same concentration as found in the plasma during the fast (5mM). VMN glucose concentrations fell during the fast, from 1 to 0.5mM. If true, this could suggest that either: a) arcuate neurons are not glucose sensing as the changes in glucose concentration they are exposed to are not an accurate representation of plasma glucose concentrations or, b) the brain knows that signals of increased glucose concentration from the arcuate mean that plasma glucose concentrations are falling, and so, when integrated with data from other regions, generate a coordinated output. The consequences of this interesting study require further exploration.

Non hypothalamic sites also contain glucose sensing neurons. CCK neurons within the lateral parabrachial nucleus were demonstrated recently, using electrophysiology, to be a glucose inhibited (Garfield et al., 2014). Using chemogenetics, these neurons were shown to regulate counter regulatory responses to hypoglycaemia, via projections to VMN SF1 neurons.

Glucose excited and inhibited neurons have been identified in the medial amygdala nucleus (Zhou et al., 2010), using calcium imaging, and functionally this brain region regulates counter regulatory responses. Just under half of the glucose sensing neurons express GK, meaning some sense glucose via other mechanisms (Zhou et al., 2010). Finally, limited electrophysiological evidence suggests glucose responsive neurons are found in the hindbrain (Adachi and Kobashi, 1985; Balfour et al., 2006; Oomura, 1983). The hindbrain is an important site in controlling the body's response to glucoprivation (Ritter et al., 2011), but may not be a principal site for primary sensing site of low glucose concentrations.

Glucose sensing sites in the brain also receive inputs from peripheral glucose sensing sites (neural and hormonal) (Hevener et al., 2001; Könnner et al., 2007; Marty et al., 2007), enabling the brain to coordinate responses when glucose concentrations change, although brain glucose sensing in the VMH (as discussed in section 1.3.1) may be able to partially override these signals (Borg et al., 1997, 1995).

1.5 How Hypothalamic Nutrient Sensing controls physiology

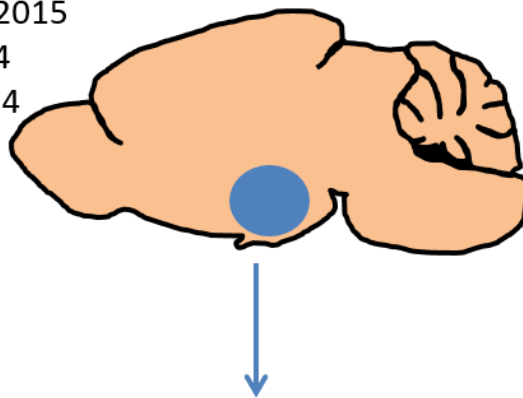
Changes in concentration of glucose and amino acids in the mediobasal hypothalamus (MBH: arcuate and ventromedial nuclei) can exert profound effects on physiology. To date, the physiological effects controlled by direct nutrient sensing in the hypothalamus have been more extensively investigated for glucose than for leucine; these effects are summarised in Figure 1-4 and Figure 1-5.

A) Low Leucine

Leib and Knight 2015

Laeger et al 2014

Maurin et al 2014



Aversive response to food devoid of leucine

B) High Leucine

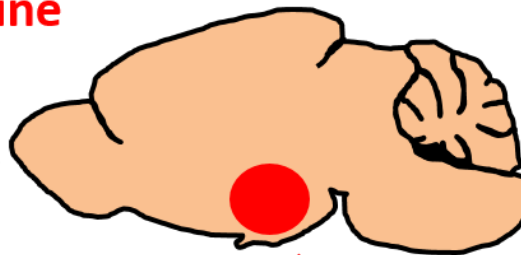
Cota et al 2006

Blouet et al 2009

Purpera et al 2012

Su et al 2012

Burke et al 2017



↓ Food intake

↓ HGP

↑ BAT
Activity

↓ Meal
size

↑ First meal
latency

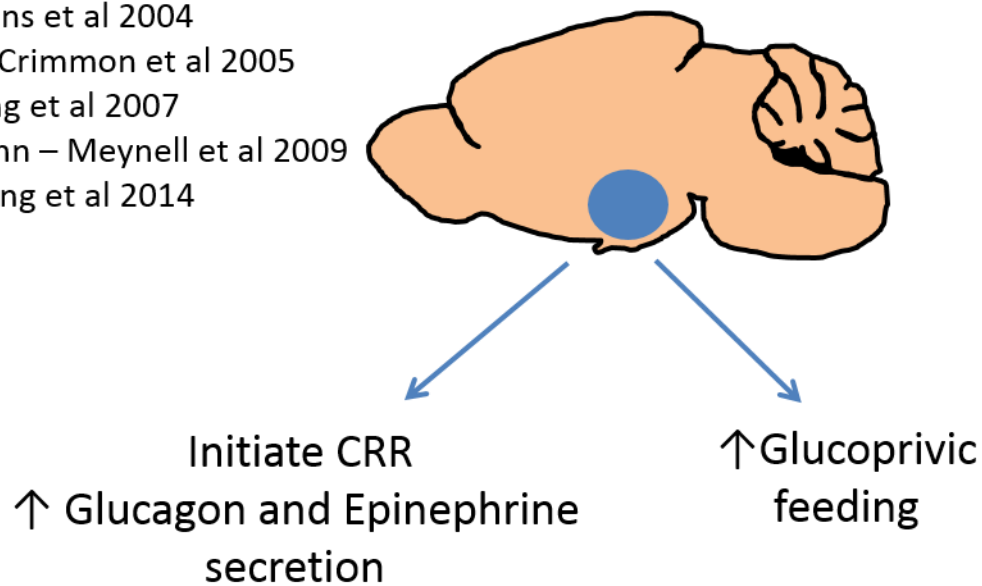
↓ Meal
number

Figure 1-4: Summary of the effects of low and high hypothalamic leucine concentrations on physiology.

A) Effects of low hypothalamic leucine concentrations and B) Effects of high hypothalamic leucine concentrations, on physiology.

A) Low Glucose

Evans et al 2004
McCrimmon et al 2005
Tong et al 2007
Dunn – Meynell et al 2009
Wang et al 2014



B) High Glucose

Parton et al 2007
Claret et al 2007
Osundiji et al 2012
Toda et al 2016

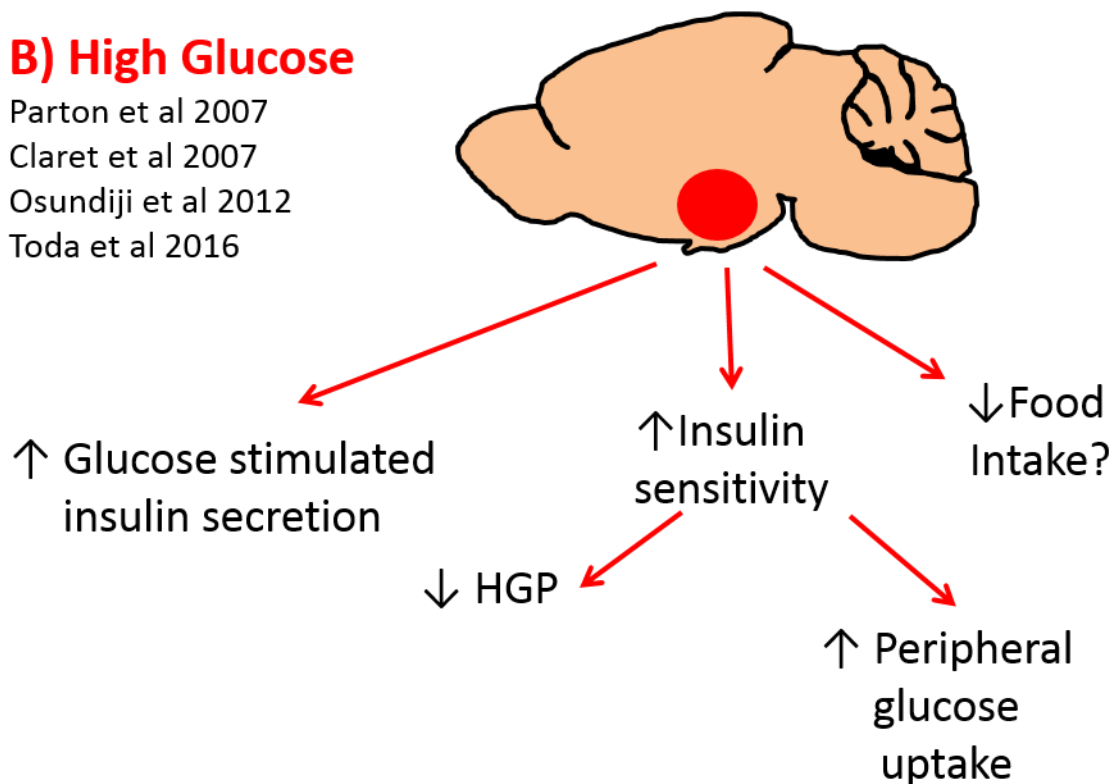


Figure 1-5: Summary of the effects of low and high hypothalamic glucose concentrations on physiology.

A) Effects of low hypothalamic glucose concentrations and B) Effects of high hypothalamic glucose concentrations, on physiology.

1.5.1 Effects of mediobasal hypothalamic leucine sensing on physiology

As discussed previously (Section 1.4.2), low amino acid concentrations in the MBH may play a role in mediating the aversive response to diets devoid of leucine, when mice are the fasted or have been amino acid deprived (Maurin et al., 2014), although these studies require further confirmation (Leib and Knight, 2015).

Leucine can acutely reduce food intake and body weight upon i.c.v. (Cota et al., 2006; Laeger et al., 2014b), MBH (Blouet et al., 2009) or NTS (Blouet and Schwartz, 2012; Cavanaugh et al., 2015) injection, and can reduce body weight when regularly injected via minipump into the MBH (Blouet et al., 2009). Leucine can control the meal sequence at multiple levels, as summarised in Figure 1-4B. MBH leucine injection suppressed hepatic glucose production (Su et al., 2012), which requires leucine metabolism, and is mTOR independent. Recent work from our lab has demonstrated that MBH leucine injection in fasted mice increases iBAT temperature, although this did not result in changes in energy expenditure (Burke et al., 2017). This effect that may be mediated via AgRP/NPY neurons. Collectively, these studies show that leucine rises in the bloodstream postprandially, likely act in the MBH to suppress feeding, and to control functions necessary for the fast refeed transition.

There is no evidence at present to suggest that the hyperphagia associated with transition to a low protein diet is mediated through sensing of lower amino acid concentrations in the MBH (Laeger et al., 2014b). Instead, peripheral signals such as secretion of FGF21 from the liver appear to mediate this effect (Laeger et al., 2014a), discussed further in Heeley and Blouet, 2016. Likewise, no direct evidence supports a role for hypothalamic leucine sensing in the control of short term hypophagia associated with transition to a high protein diet. This suggests leucine, acting in the MBH can exert short term effects on physiology and behaviour, but longer term effects attributed to MBH amino acid sensing have not been demonstrated. The longer term, and well described effects of changes in protein abundance (so called protein leverage (Simpson and Raubenheimer, 2005; Simpson et al., 2015; Solon-Biet et al., 2014)) are likely due to peripheral signals which may act in the brain (Laeger et al., 2014a).

1.5.2 Effects of mediobasal hypothalamic glucose sensing on physiology

1.5.2.1 Controlling the counter regulatory response to hypoglycaemia

The best-characterised effect is that lowering brain glucose concentration is particularly important in the initiation of the CRR (Beall et al., 2012; Dunn-Meynell et al., 2009; Oomura et al., 1969; Vries et al., 2003). It has been proposed that the MBH acts as the integrator of glucose – sensor signals from both local and peripheral sources (Sherwin, 2008), and is the site from which the CRR is initiated. Neurons in many brain regions *ex vivo* have been shown to be glucose sensing (see section 1.4.3), and recent studies have begun to explore how these neurons work together *in vivo* to mediate, the counter regulatory responses to hypoglycaemia.

A recent study selectively activated glucokinase (GK) expressing neurons in the VMN that Cre dependently expressed an ion channel engineered to open or close (depending upon the ion channel used) upon exposure to a strong magnetic field (Stanley et al., 2016). Activation of VMN GK neurons using this method, or using optogenetics, increased blood glucose concentrations, with inhibition having the opposite effect (Stanley et al., 2016). Activation halved plasma insulin concentrations, increased plasma glucagon concentrations, and increased hepatic glucose – 6 – phosphatase (necessary for gluconeogenesis) gene expression, all components of the CRR to hypoglycaemia. A similar study demonstrated that optogenetically inhibiting VMN SF1 neurons caused blood glucose levels to fall more during an insulin tolerance test; activation of the same neurons enhanced increases in blood glucose concentrations during a pyruvate or glucose tolerance test (Meek et al., 2016). Similar effects were reported on CRR hormone production and hepatic gene expression, as in Stanley et al., 2016. Supporting a previous study (Garfield et al., 2014), circuit mapping demonstrated that VMN SF1 neurons received inputs from glucose sensitive CCK neurons in the LPBN (Meek et al., 2016), and project to the aBNST to initiate these effects, a region where AgRP/NPY neurons are known to project to control glucose homeostasis through changes in BAT insulin sensitivity (Steculorum et al., 2016).

Given the similar phenotypes observed when activating VMN SF1 and GK expressing neurons, it is likely that many SF1 VMN neurons express glucokinase, and may sense glucose via this mechanism. Mice generated in a recent study, where GK was knocked out from VMN SF1 neurons, had reduced glucagon secretion in response to hypoglycaemia and reduced

parasympathetic and sympathetic nerve activation monitored *in vivo* in response to glucoprivation (i.p. 2DG) (Steinbusch et al., 2016). However, these effects were only observed in female mice. The previous studies (Meek et al., 2016; Stanley et al., 2016) do not clearly state which gender is used. Interestingly, SF1 GK KO neurons were still able to alter action potential firing in response to changes in glucose concentrations, when assessed by electrophysiology, questioning the role glucokinase plays in glucose sensing in these neurons.

1.5.2.2 Controlling feeding behaviour

Adenoviral mediated knockdown of glucokinase in the MBH of rats reduces glucoprivic feeding (Dunn-Meynell et al., 2009), demonstrating a) the MBH is an important site for initiating this response and b) that metabolism dependent sensing mechanisms are involved. Neonatal ablation of AgRP/NPY neurons in the MBH did not alter glucoprivic feeding (Luquet et al., 2007), but in this model, feeding is maintained by unknown compensatory mechanisms (Luquet et al., 2005, 2007), whereas ablation of AgRP/NPY neurons in adulthood results in starvation (Luquet et al., 2005). This suggests compensatory changes may allow glucoprivic feeding to continue in adults, and that in normal mice, AgRP/NPY neurons may be necessary for the response. Feeding was not assessed when SF1 VMN neurons were optogenetically activated or inhibited (Meek et al., 2016), or in SF1 GK knock out mice (Steinbusch et al., 2016). Activating glucokinase expressing neurons in the VMN, increased food intake, and when inhibited, had an anorectic effect (Stanley et al., 2016). This suggests that these neurons may be involved in the control of glucoprivic feeding, although glucoprivic feeding was not specifically tested.

Although hypoglycaemia undoubtedly induces a feeding response, whether brain glucose sensing regulates food intake is an area of long debate in the field. The role of a fall in glucose concentration in meal initiation is not well supported (Dunn-Meynell et al., 2009), although a study where GK expression was manipulated in the arcuate nucleus of rats showed GK knockdown reduced glucose and food intake, and intake was increased when GK was overexpressed in the arcuate nucleus (Hussain et al., 2015). These recent studies (Hussain et al., 2015; Stanley et al., 2016) provide some evidence that glucose sensing neurons may play a role in feeding behaviour, depending on when these GK expressing neurons are activated during normal physiology. Other groups have also been able to show that glucose can suppress food intake, and that this effect is linked to the metabolism of

glucose to malonyl - CoA (likely involving the K_{ATP} channel) (Cha et al., 2008; Wolfgang et al., 2007).

1.5.2.3 Control of peripheral glucose homeostasis

Additionally, hypothalamic glucose-sensing may regulate peripheral glucose homeostasis (Parton et al., 2007; Toda et al., 2016). Knock out of the Kir6.2 subunit (necessary for functional K_{ATP} channels) from POMC neurons, resulted in a larger glucose excursion during a glucose tolerance test. POMC neurons from KO mice were glucose insensitive, as assessed by electrophysiology. POMC neurons from high fat diet fed mice were also glucose insensitive; UCP2 (uncoupling protein 2) deletion prevented this effect. Deletion of DRP1 (dynamin-related protein 1)(that lies downstream from UCP2 and is necessary for mitochondrial fission) from POMC neurons of adult mice, improved responses to insulin induced hypoglycaemia, through increased glucagon secretion (Santoro et al., 2017). Further work in SF1 VMN glucose excited neurons has proposed a link between UCP2 dependent glucose sensing and K_{ATP} channel activation in the control of peripheral insulin sensitivity (Steculorum and Bruning, 2016; Toda et al., 2016), suggesting the VMN controls general glucose homeostasis, as well as the CRR.

Studies from our lab have also shown that glucose stimulated insulin secretion is enhanced when glucose is infused into the third ventricle, but was reduced when glucokinase inhibitors are infused (Osundiji et al., 2012).

So MBH glucose sensing can regulate glucose homeostasis and feeding behaviour at multiple levels. Integration with peripheral signals also sensed by this region, such as leptin, insulin and gut hormones, allow a coordinated output to be generated in response to changing metabolic state (Kohno et al., 2003; Könner et al., 2007; Murphy et al., 2009a; Routh et al., 2014; Wang et al., 2014).

1.5.3 Diseases related to defective nutrient sensing

Dysregulation of branched chain amino acid uptake into the brain has been linked to diseases such as Maple Syrup Urine Disorder (MSUD) and Autism Spectrum Disorder (ASD) (Tărlungeanu et al., 2016; Yudkoff et al., 2005). Patients with MSUD have whole body mutations in BCKDC (Branched-chain alpha-keto acid dehydrogenase complex), an enzyme necessary for controlling BCAA catabolism, resulting in high plasma BCAA concentrations (Fernstrom, 2005; Yudkoff et al., 2005). High brain leucine concentrations are neurotoxic.

This is due to osmotic dysregulation and disruption of uptake of other amino acids into the brain (Fernstrom, 2005; Maynard and Manzini, 2017; Muelly et al., 2013). A recent study showed that mutations in LAT1 (large neutral amino acid transporter 1) or BCKDK (Branched chain ketoacid dehydrogenase kinase) resulted in ASD (Tărlungeanu et al., 2016). LAT1 is a membrane amino acid transporter, that transports leucine (and other amino acids) into cells. BCKDK inactivates BCKDC, reducing BCAA metabolism. These mutations, in LAT1 and BCKDK, had the opposite effect to MSUD, but were equally damaging to the brain, again due to dysregulated amino acid uptake into the brain. Modelling the disease through LAT1 KO in endothelial cells of the blood brain barrier, to alter brain amino acid uptake, demonstrated that the phenotypes were mediated in part through activation of the GCN2 signalling pathway, but not through changes in mTORC1 (Tărlungeanu et al., 2016). This suggests that novel pathways may be involved, or that the analyses conducted, principally at the transcriptional level, did not capture alterations caused by dysregulation of mTORC1 signalling (highlighted by Maynard and Manzini, 2017). It may be the case that these diseases are due to the absence of nutrient sensing into the brain, although this link has not been proven.

Dysregulation of brain glucose sensing may be involved in the generation of HAAF during intensive insulin therapy to treat diabetes (Ogunnowo-Bada et al., 2014; Sherwin, 2008). Recurrent hypoglycaemia has been widely reported to reduce counter regulatory responses to hypoglycaemia (Beall et al., 2012; McCrimmon et al., 2005; Sherwin, 2008; Song and Routh, 2006; de Vries et al., 2003). The teleological basis of altered hypoglycaemia sensing is not fully understood (Beall et al., 2012; Ogunnowo-Bada et al., 2014; Sherwin, 2008), but appear to be due to the brain's attempts to defend itself against future hypoglycaemia by altering neuronal metabolism to allow alternative fuels to be used in future hypoglycaemic bouts. Blood and MBH glucose concentrations are lower during recurrent hypoglycaemic bouts (Song and Routh, 2006; de Vries et al., 2003); a reduction in glucose sensitivity by VMH glucose inhibited neurons may be a contributory factor (Song and Routh, 2006). Defective glucagon responses in many diabetic patients (Beall et al., 2012; Ogunnowo-Bada et al., 2014) means the counter regulatory response that is mounted may be less effective than normal.

Nutrient sensing has been shown to be disrupted during obesity (Cavanaugh et al., 2015; Parton et al., 2007; Song et al., 2001). Leucine's anorectic effect when injected into the NTS

is abolished in mice fed a high fat diet for 6 months (Cavanaugh et al., 2015). Leucine's affect to suppress appetite over 24 hours after 2 week exposure to a high fat diet was maintained, although the acute effects on first meal latency and size were abolished with the high fat diet. As discussed earlier (section 1.5.2), in POMC neurons, glucose sensing is lost when mice are placed on a high fat diet (Parton et al., 2007).

These studies demonstrate that dysregulation of brain nutrient sensing causes serious health problems. Functional brain nutrient sensing therefore, is essential to maintain health.

Multiple mechanisms (some already discussed) have been identified by which nutrients are sensed in the MBH, which are discussed in the next section.

1.6 Heterogeneity in hypothalamic nutrient sensing mechanisms

1.6.1 Leucine sensing mechanisms in the MBH

Multiple signalling pathways in the MBH have been demonstrated to sense leucine, summarised in Figure 1-6. These pathways, through pharmacological and genetic manipulations in the MBH, have been shown to control physiology and behaviour. However, the link between leucine induced changes in signalling through these pathways and changes in neuronal function are less clear.

1.6.1.1 Mechanisms sensing low amino acid concentrations

The GCN2 pathway is activated by uncharged tRNA synthetases, which accumulate when cellular amino acid concentrations are low, initiating a signalling cascade that inhibits cellular translation, except for pathways involved in amino acid biosynthesis which are upregulated (Chantranupong et al., 2015; Morrison and Laeger, 2015). As discussed previously, low leucine concentrations, in specific metabolic situations, can be sensed via the GCN2 pathway (Gietzen et al., 2016; Hao et al., 2005; Maurin et al., 2005, 2014), although further studies are required to strengthen the evidence showing that this happens in the MBH (Maurin et al., 2014). Other pathways, such as Erk1/2 signalling (Sharp et al., 2006), intracellular calcium signalling (Magrum et al., 1999), and membrane amino acid transporters (Blais et al., 2003), have been shown to play a role in sensing of low amino acid concentrations in the APC, but not yet in the MBH.

1.6.1.2 Metabolism independent leucine sensing pathways: mTORC1

Several signalling pathways have been implicated in sensing high MBH leucine concentrations. The mTORC1 complex is an important integrator of multiple signals of nutrient and energy status (Chantranupong et al., 2015). These factors alter either the activation state of mTORC1 or its subcellular localisation, both necessary for pathway activation. The mTORC1 pathway coordinates transcription, translation, cell growth, cell cycle progression, and other processes (Heeley and Blouet, 2016; Laplante and Sabatini, 2012). Leucine potently activates the mTORC1 pathway, which cellular studies have shown is through leucine binding to Sestrin 2, which represses repressors of the mTORC1 complex, resulting in mTORC1 activation (Wolfson et al., 2016). Sestrin 2 has not yet been implicated in MBH leucine sensing. mTORC1 has previously been implicated in leucine's ability to control food intake. Cota et al., 2006 showed that i.c.v. injection of leucine could reduce food intake and body weight over 24 hours. This was accompanied by an increase in MBH mTORC1 activity (higher phosphorylated mTOR and p70 S6K1, a key downstream effector) which was blocked by the injection of rapamycin, an inhibitor of mTORC1. Bidirectional manipulation of the expression of p70 S6K1, in the MBH modulates food intake, body weight and energy expenditure (Blouet et al., 2008). S6K1 knock out from POMC neurons prevented leucine induced increases in action potential firing, although a supraphysiological leucine concentration was used in this study (Smith et al., 2015). The effectors downstream of p70 S6K1 signalling that control the function of leucine sensing neurons have not been explored.

1.6.1.3 Metabolism independent leucine sensing pathways: Erk1/2

Erk1/2 has been shown to be a necessary mediator of some of the effects of leucine in the MBH (Blouet et al., 2009). Co – injection of an inhibitor of a kinase downstream of Erk1/2 into the MBH with leucine, blunted leucine's short term anorectic effect, restoring first meal size, 24 hour food intake and body weight change to levels comparable to ACSF controls. This suggests that Erk1/2 activation is necessary for leucine's anorectic effect, but as for mTORC1 signalling, the link between factors downstream of Erk1/2 signalling in leucine sensing neurons have not been studied.

1.6.1.4 Metabolism dependent leucine sensing pathways

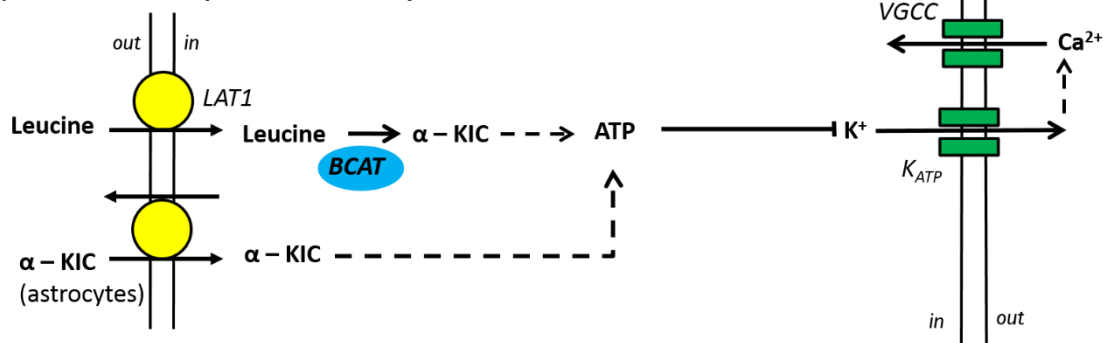
Leucine may also be sensed through its metabolism by a mechanism that may converge on the K_{ATP} channel (or AMPK but this has not been studied), as for metabolism dependent glucose sensing (discussed further in section 1.6.2). Su and colleagues demonstrated that

leucine metabolism converges on K_{ATP} channels to suppress hepatic glucose production after MBH leucine injection (Su et al., 2012). α – KIC (alpha-ketoisocaproate), the first metabolite of leucine, mimics the anorectic effect of MBH leucine injection, and chronic MBH administration of CIC (alpha chloro-isocaproic acid), resulting in chronic BCAT activation, suppresses food intake via an effect on meal number (Blouet et al., 2009).

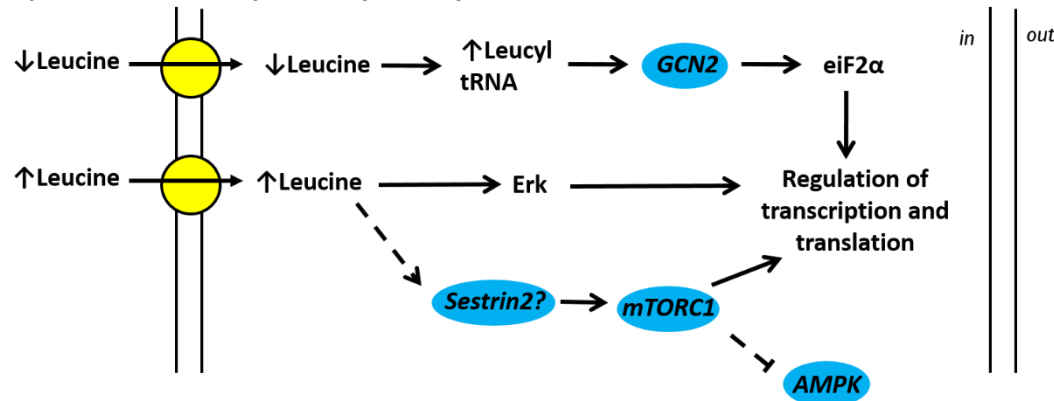
1.6.1.5 How do leucine sensing pathways control neurophysiology?

Controlling K_{ATP} channel activity is the only clear mechanism identified by which leucine may directly regulate action potential firing. The GCN2, Erk and mTORC1 pathways control transcription and translation, thus influencing cellular function, which can influence neurotransmission; for instance, mTORC1 signalling has been demonstrated to alter AgRP/NPY expression in a hypothalamic cell line (Morrison et al., 2007). However, the time course of leucine dependent effects on feeding, shown molecularly and pharmacologically to be mediated by these pathways, is likely too fast to be due to changes in transcription and translation controlled by these pathways. This suggests that either, 1) these pathways must be able to rapidly modulate action potential firing of MBH neurons through an as yet undescribed mechanism or 2) changes in transcription and translation, through emerging pathways such as through reactivating stalled polysomes may be responsible for leucine's effects (Graber et al., 2013; Pimentel and Boccaccio, 2014), or 3) these pathways can influence neuronal function via other mechanisms. It must be noted that leucine sensing via the mTORC1 pathway does not exert rapid effects on feeding behaviour in the MBH, but it does in the NTS, so this question still needs addressing (see table on speed of effects of MBH leucine in introduction, chapter 2). Further work is needed to fully understand the mechanisms of action of MBH leucine.

A) Metabolism Dependent Pathways



B) Metabolism independent pathways



C) Other Amino acid sensing pathways, not implicated in MBH amino acid sensing:

Tas1r1/r3

GDH

LAT1

Figure 1-6: Leucine sensing pathways in the MBH.

A) Metabolism dependent leucine sensing pathways implicated in MBH leucine sensing. α -KIC, from leucine metabolism via BCAT to form or direct α -KIC transport into neurons from astrocytes, is metabolised, producing ATP. ATP closes K_{ATP} channels, which leads to membrane depolarisation and opening of VGCC. B) metabolism independent leucine sensing pathways implicated in MBH leucine sensing. Reduction in leucine concentrations lead to an increase in leucyl tRNA abundance, which activates GCN2, leading to a suppression of translation. Increases in leucine concentration are sensed by the mTORC1 pathway, which regulates transcription and translation, and can inhibit AMPK activity. C) Other amino acid sensing pathways implicated in the periphery, but not the MBH. Dashed lines indicate connections that are hypothesised, but not proven. α -KIC - alpha-ketoisocaproate, AMPK - 5' AMP-activated protein kinase, ATP - adenosine triphosphate, BCAT – branched chain aminotransferase, eIF2 α – eukaryotic initiation factor 2 α , Erk - Extracellular signal–regulated kinases, GCN2 - general control nonderepressible 2, GDH – glutamate dehydrogenase, LAT1 – large neutral amino acid transporter 1, mTORC1 - mammalian target of rapamycin complex 1, Tas1r1/3 - Taste receptor type 1 members 1 and 3, tRNA – transfer RNA, VGCC – voltage gated calcium channel

1.6.2 Glucose sensing mechanisms in the MBH

Glucose sensing mechanisms known to exist within the MBH are summarised in Figure 1-7. Multiple pathways dependent upon the metabolism of glucose have been implicated in MBH glucose sensing. Clearer links have been established between changes in glucose induced signalling through these pathways and changes in action potential firing, the classical way a glucose responsive neuron has been identified (Oomura et al., 1969), although it is likely that glucose acts as a signalling molecule in other neurons, that are not glucose sensing by the classical definition, as assessed by electrophysiology or calcium imaging.

1.6.2.1 Metabolism dependent glucose sensing: pancreatic beta cell mechanism

It is proposed that as in the beta cell, in neurons: glucose enters the cell via protein transporters (GLUT2); it is phosphorylated by glucokinase, to form glucose – 6 – phosphate producing ATP which regulates the gating of K_{ATP} channels in GE neurons (Figure 1-7A). Electrophysiology studies have shown the necessity of key components of this pathway for glucose sensing, such as the K_{ATP} channel (Miki et al., 2001), that also plays a role in leucine sensing (Su et al., 2012). Some of the proteins necessary for glucose sensing, such as GK, GLUT transporters and K_{ATP} channel subunits have been shown to be co – expressed in some GE neurons (Dunn-Meynell et al., 2002; Kang et al., 2004; Miki et al., 2001), however, not all GE neurons express these proteins, and they are also expressed in non – glucose sensing neurons (González et al., 2009; Kang et al., 2004; Lynch et al., 2000; Sanz et al., 2011), suggesting this is not the only glucose sensing mechanism in GE neurons. Additionally, a study monitoring intracellular ATP concentrations in glucose excited neurons did not observe changes in ATP concentrations in response to glucose application (0 to 3mM increase) (Ainscow et al., 2002). However, these studies were performed on neurons from early postnatal rats, cultured for 6 – 9 days, in media with FBS and high glucose concentrations, all of which may alter the glucose sensing properties of neurons (Kang et al., 2006) compared to adult MBH glucose sensing *in vivo*.

1.6.2.2 Metabolism dependent glucose sensing: AMPK and Nitric Oxide

Some studies have suggested that glucose inhibited neurons may sense glucose using the beta cell like pathway (Dunn-Meynell et al., 2002), but others have proposed that Nitric Oxide, produced downstream of AMPK in response to falls in glucose concentrations, leads to changes in the opening of the CFTR chloride channel, is the glucose sensing mechanism in glucose inhibited MBH neurons (Fioramonti et al., 2010; Murphy et al., 2009b). AgRP/NPY

neurons are MBH glucose inhibited neurons, but sense glucose by a different mechanism (Hao et al., 2016).

1.6.2.3 Metabolism dependent glucose sensing: UCP2

Glucose sensing in POMC, AgRP, and SF1 VMN GE neurons (Dietrich et al., 2013; Santoro et al., 2017; Toda et al., 2016), has been shown to involve mitochondrial ROS (reactive oxygen species) production. A recent study of VMN SF1 neurons showed that increasing hypothalamic glucose concentrations induced changes in mitochondrial fission, dependent upon UCP2 induced phosphorylation of DRP1, leading to decreased ROS production (Toda et al., 2016). Neuronal activation was dependent upon K_{ATP} channels closure, although the functional link between ROS production and K_{ATP} channels was not assessed (Toda et al., 2016). Whether K_{ATP} channel closure in previous studies (e.g.: (Kang et al., 2004)) of glucose excited neurons is mediated by this UCP2 dependent pathway is unknown. Unusually, DRP1 knock out from POMC neurons in adult mice resulted in a gain of glucose sensing function, increasing the number of glucose sensing POMC neurons (Santoro et al., 2017). In the arcuate nucleus of knock out mice, *Kcnj11* (encoding the Kir6.2 subunit of K_{ATP} channels) gene expression was increased, mediated by PPAR γ , altering the ability of neurons to sense glucose (Santoro et al., 2017). DRP1 activation by phosphorylation, is regulated by feeding, suggesting this is a mechanism relevant to glucose sensing in normal physiology (Santoro et al., 2017). This data suggests that glucose sensing can act over the shorter term, by altering neuronal activation (e.g.: K_{ATP} channels, (Toda et al., 2016)), and in the longer term, by altering gene expression and mitochondrial dynamics (Santoro et al., 2017), to impact neuronal dynamics and impact future nutrient sensing.

1.6.2.4 Metabolism independent glucose sensing

However, others have suggested that a metabolism dependent sensor would only be useful to respond to emergency situations of extremely low nutrient availability e.g.: hypoglycaemia (González et al., 2009). They propose that other sensors that aren't dependent on metabolism are more important in the control of energy homeostasis, when glucose concentrations are within the physiological range. Work from the groups proposing this hypothesis have outlined possible metabolism independent glucose sensing mechanisms.

In rat hypothalamic neuron cultures, SGLT1/2, glucose transporters, were shown to be necessary for glucose induced calcium responses (O'Malley et al., 2006). Single cell RT – PCR demonstrated these transporters were expressed in hypothalamic glucose excited neurons. However, it must be noted that in this study, neurons were cultured in media containing 17.5mM glucose. Additionally, some neurons were glucose excited using a K_{ATP} dependent mechanism. Imaging was performed a minimum of 24 hours after culture, and in some cases up to six days post extraction, a timeframe when glucokinase expression reduces significantly in neuron cultures (Kang et al., 2006). This suggests a larger fraction of neurons may normally sense glucose via a metabolism dependent mechanism. SGLT's could be upregulated during the culture, and may in this study account for more glucose sensing neurons than *in vivo*. This study was performed on whole rat hypothalamus, so it is not clear from this study whether MBH glucose sensing is SGLT dependent.

Studies of lateral hypothalamic glucose inhibited orexin neurons (Gonza et al., 2008), and unidentified MBH glucose inhibited neurons (Williams and Burdakov, 2009), have demonstrated that these neurons sense reduced glucose concentrations by closure of an outwardly rectifying potassium channel. High glucose concentrations result in hyperpolarisation of these neurons. The identity of the potassium channel is unknown (Burdakov and Lesage, 2010). The mechanism by which glucose is sensed upstream of channel closure is not known, but is mimicked by non metabolisable glucose analogues (so is metabolism independent) (Gonza et al., 2008).

A recent study from the same group suggested this metabolism independent mechanism was regulated by cellular energy status (Venner et al., 2011). Low cellular energy status (tested using metabolic poison oligomycin) enhanced glucose inhibition of orexin neurons, and high cellular energy status (tested by pyruvate or lactate injection into cells during recordings) suppressed glucose responses, suggesting an interplay between the metabolic state of the cell, and metabolism independent glucose sensing (Venner et al., 2011). It could be argued that metabolism dependent energy sensing mechanisms permit metabolism independent glucose sensing. Similarly, in AgRP/NPY neurons, low cellular energy status, after fasting, enhances the response of AgRP/NPY neurons to glucose, i.e.: the same fall in glucose concentration causes a larger depolarisation in the fasted state (Murphy et al., 2009a). This

mechanism may be AMPK dependent. In keeping with this, maintaining VMH GI neurons in hyperglycaemic conditions blunts the response of these neurons to glucose (Canabal et al., 2007a).

A subpopulation of glucose sensing orexin neurons possess a property called adaptation (Williams et al., 2008), analogous to that found in sensory systems, such as the eye. Prolonged glucose application to orexin neurons resulted in a subpopulation of orexin neurons resuming firing after initial hyperpolarisation, monitored by electrophysiology in slice preparations. Further support for this observation came from double step experiments, where glucose concentrations were increased twice, with adaptation occurring after the first change in glucose concentration, allowing the neuron to sense the second change in glucose concentration (Williams et al., 2008). From the new, adapted baseline, after the first step, glucose fluctuations can still be sensed. This allows orexin neurons in this subpopulation to respond to glucose concentration changes across the whole physiological range. This effect is mediated by closing potassium leak channels, which are opened in response to high glucose concentrations (Figure 1-7B), but why this occurs in this subpopulation of orexin neurons but not in non-adapting neurons is unclear, given that they both utilise the same potassium channel to respond to glucose. Adaptation was not observed when experiments were conducted at room temperature. Orexin neurons control arousal, and so the authors hypothesise this mechanism may be necessary to maintain wakefulness postprandially when blood glucose concentrations rise (Williams et al., 2008). The smaller, non-adapting population of orexin neurons sense absolute changes in blood glucose concentrations. The adapting and non-adapting orexin subpopulations had different spatial distributions. It is not clear if this adaptive glucose sensing is linked, or is independent from the effects of metabolic state on glucose inhibited neurons that the same group identified (Venner et al., 2011). Adapting glucose inhibited neurons have been identified recently in the VMH (Santiago et al., 2016a, 2016b).

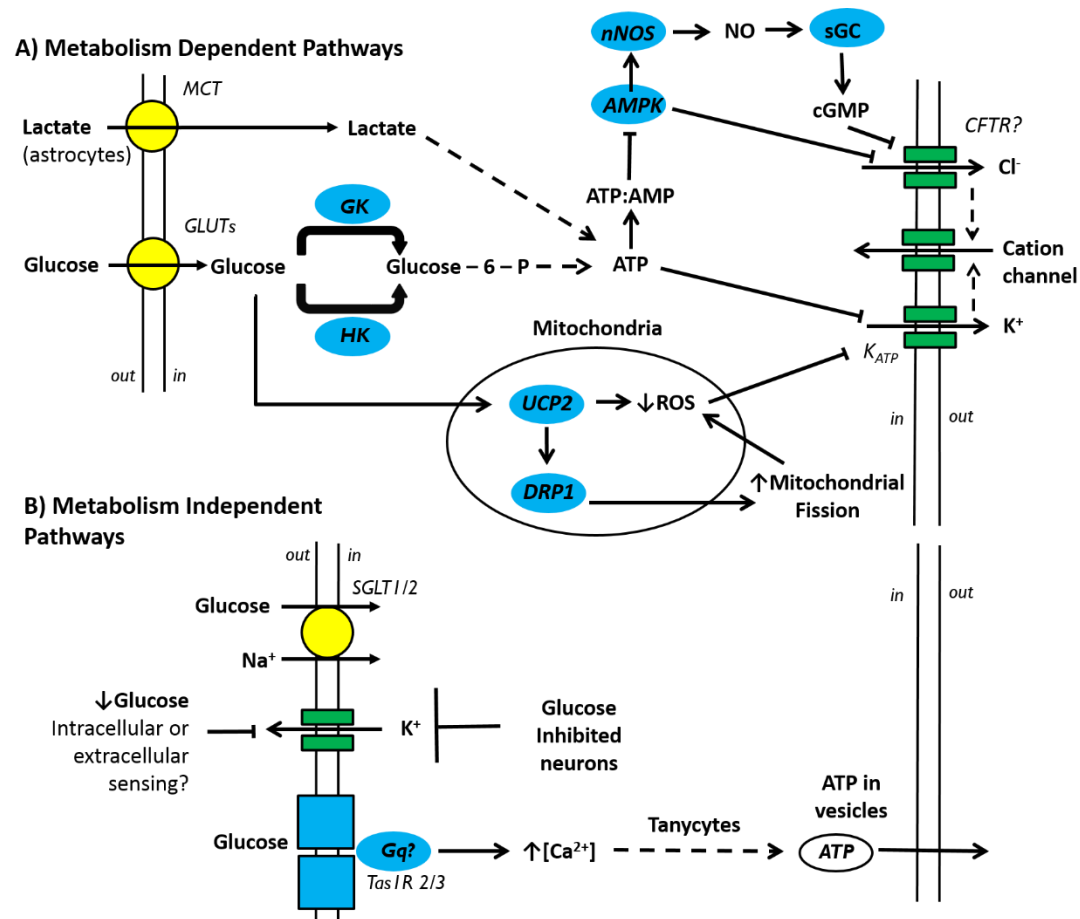


Figure 1-7: Glucose sensing pathways in the mediobasal hypothalamus.

A) Metabolism dependent glucose sensing pathways in the MBH. Lactate or glucose metabolism produces ATP, which can be sensed by neurons via K_{ATP} channel closure and subsequent membrane depolarisation, or via an AMPK/nNOS/CFTR dependent pathway. Glucose via a UCP2 dependent pathway, resulting in mitochondrial fission and/or K_{ATP} channel closure. B) Metabolism independent glucose sensing pathways in the MBH. Glucose may be sensed by the electrogenic transporter, the Taste receptor dimer Tas1r2/3, or an as yet unidentified membrane potassium channel (Burdakov and Lesage, 2010; Hao et al., 2016). AMPK - 5' AMP-activated protein kinase, CFTR – cystic fibrosis transmembrane conductance regulator, DRP1 - dynamin-related protein 1, GK – glucokinase, glucose – 6 – P – glucose 6 phosphate, GLUT - glucose transporter, Gq – G protein type q, HK – hexokinase, MCT - Monocarboxylate transporter, nNOS – neuronal nitric oxide synthase, NO – nitric oxide, ROS – reactive oxygen species, sGC – soluble guanylyl cyclase, SGLT – sodium glucose transporter, UCP2 – uncoupling protein 2. Dashed lines indicate connections that are hypothesised, but not proven.

1.6.3 Why do multiple nutrient sensing mechanisms exist?

Although multiple sensing mechanisms exist, what is their purpose? There are several possible explanations. Firstly, this could reflect the need for redundancy in nutrient sensing. For some functions, such as sensing hypoglycaemia, multiple mechanisms may be used, as severe hypoglycaemia is deadly, so defending against it is essential for survival. Multiple mechanisms can defend against error in one mechanism.

Some sensors may perform different functions. For instance, GCN2 is specialised for sensing low amino acid concentrations. It is not suited to sense increases in amino acid concentrations. Additionally, pathways such as mTOR and AMPK serve as hubs for multiple signalling pathways, whereas more specialised signalling mechanisms, such as SGLT or GK mediated glucose sensing, are specific for a single nutrient. Signals from SGLT or GK mediated sensing could be integrated by pathways such as mTOR or AMPK to control cellular function. However, understanding how these signalling pathways interact is challenging to study, particularly *in vivo*.

Some candidate sensing mechanisms may not be true glucose sensing mechanisms *in vivo*. For instance, glucose sensing via SGLT transporters, demonstrated to occur in rat hypothalamic neurons *ex vivo* (O'Malley et al., 2006), has not been shown to affect physiology. While a nutrient sensing mechanism may exist, without controlling known physiological effects, it is unclear what its role is.

While studies have identified multiple nutrient sensing mechanisms in the MBH, and multiple mechanisms by which nutrients in the MBH regulate physiology and behaviour, the links between these sensing pathways and the control of the neurophysiology of neurons have not been fully addressed. In addition, the neural circuits mediating these effects, require further exploration. While the studies discussed have focussed on neuronal nutrient sensing, a growing literature suggests other hypothalamic cell types may be nutrient sensing.

1.7 Heterogeneity in hypothalamic cell types responsible for Nutrient Sensing

Neurons have been the primary focus of studies examining hypothalamic nutrient sensing. Numerous studies have demonstrated a role for neurons in glucose sensing, with the

existence of glucose excited and inhibited neurons, sensing glucose by multiple mechanisms. However, other cell types in the MBH may also play a role in nutrient sensing.

1.7.1 Astrocytes

There is a growing body of work demonstrating a role for astrocytes in nutrient sensing. Astrocytes may be a site where leucine sensing is initiated, as astrocytes can metabolise leucine to its first metabolite KIC, which they release to neurons, for synthesis of glutamate (Yudkoff et al., 2005). This could be a pathway by which leucine is sensed. Data shows that MBH injection of equimolar concentrations of KIC have an equivalent anorectic effect to leucine (Blouet et al., 2009), and that the suppression of hepatic glucose production by leucine requires its transamination to produce KIC (Su et al., 2012). However, there is no evidence to show that this is dependent on astrocytes at present as BCAT is expressed by both astrocytes and neurons (García-Espinosa et al., 2007).

The same study that reported a lack of ATP concentration changes in glucose excited MBH neurons (Ainscow et al., 2002), did observe changes in ATP concentrations in neurons in response to lactate application. Astrocytes were shown to metabolise glucose, principally by glycolytic metabolism, releasing lactate that caused rapid cytosolic ATP elevations in neurons (Ainscow et al., 2002). Both astrocytes and neurons express MCT1, a lactate transporter necessary for this shuttling (Ainscow et al., 2002). This suggests astrocytes may be the primary site of metabolism dependent glucose sensing in the hypothalamus. However, it was unclear from this study whether lactate induced increases in cytosolic ATP in neurons caused closure of K_{ATP} channels. Further work suggested glucose and lactate are sensed by the same neurons, with lactate not stimulating non glucose sensing VMH neurons (Song and Routh, 2005). These affects were K_{ATP} dependent. Reduced action potential firing in glucose excited neurons caused by decreased glucose was reversed by lactate, but lactate application paradoxically increased action potential firing of glucose inhibited neurons (Song and Routh, 2005).

In vivo, VMN lactate infusion has been shown to suppress hypoglycaemia counter regulatory responses (Borg et al., 2003). Increased lactate fluxes may preserve neuronal metabolism after recurrent bouts of hypoglycaemia, contributing to hypoglycaemia unawareness and hypoglycaemia associated autonomic failure (Herzog et al., 2013; Litvin et al., 2013). This data suggests that, like glucose, lactate may serve as both a fuel for neurons in the MBH, and

is sensed by them to regulate physiological processes (which may have detrimental effects on health).

Regulating astrocyte activity *in vivo* can alter energy homeostasis. Two recent studies using chemogenetics to activate astrocytes have shown that they can cause diverging effects on feeding, increasing (Chen et al., 2016) or decreasing (Yang et al., 2015) feeding via regulation AgRP neurons. This may be via the release of ATP from astrocytes, inhibiting the activity of AgRP neurons via Adenosine 1 receptors. The authors of the more recent study suggest the diverging results may be due to excessive CNO doses used in the first study causing “non – specific inhibition” of feeding (Chen et al., 2016).

1.7.2 Tanycytes

Tanycytes are an under studied population of hypothalamic cells, which are emerging as an important nutrient sensing population. As discussed earlier (section 1.4.3), glucose sensing by tanycytes appears to play an important role in remodelling the blood brain barrier during fasting, with the glucose concentration in the arcuate nucleus paradoxically increasing during a fast, although the tanycytic glucose sensing mechanism in this study was not identified (Langlet et al., 2013). Given that tanycytes are likely exposed to a larger range of glucose and amino acid concentrations compared to other hypothalamic cells, due to the privileged access that this population has to the bloodstream, tanycytes may serve as an important hypothalamic nutrient sensing population, communicating to neurons to control physiology (Frayling et al., 2011; Gao et al., 2014; Langlet et al., 2013).

Studies have examined the mechanisms related to tanycytic glucose sensing. Puffing glucose locally onto tanycytes in slice preparations evoked waves of calcium to occur across multiple tanycytes (Frayling et al., 2011). This was due to ATP release from some tanycytes evoking calcium signals in other tanycytes. The authors propose that, given the speed of the responses (within seconds), and because non metabolisable glucose analogues mimic the glucose response, that glucose sensing occurs via a metabolism independent mechanism. Further work from the same group (Benford et al., 2017) using calcium imaging, demonstrates that the majority of tanycytes sense glucose via a Tas1r2/3 (Taste receptor type 1 members 2 and 3) dependent mechanism, with only 40% of tanycytes still glucose sensing in Tas1r2 knock out mice. Glucokinase, and other components of the pancreatic beta cell glucose sensing pathway may be expressed in hypothalamic tanycytes, but few studies

have explored this further (Salgado et al., 2014; Thomzig et al., 2005). Adenoviral mediated knock down of glucokinase in rat tanycytes of the hypothalamus (but possibly other cells as the injection was directly into the third ventricle), suppressed food intake, suggesting glucokinase mediated glucose sensing may control feeding via tanycytes in the hypothalamus (Uranga et al., 2017).

In summary, the study of nutrient sensing must not be limited to neurons, but must also consider tanycytes and astrocytes. How do these different populations function together to coordinate nutrient sensing in the MBH?

1.8 Subpopulations within known neuronal populations

Not all neurons in the MBH are nutrient sensing. Some have suggested that the nutrient sensing neurons that do exist may be “metabolic sensing neurons”, that act as integrators of multiple nutritional signals, and are not just nutrient sensing (Levin, 2002, 2006; Routh, 2002). How nutrient and hormone sensing mechanisms are organised in the MBH is an under explored area, but may be aided by a growing understanding of the heterogeneity and complexity of the MBH.

1.8.1 Subpopulations determined based on function

POMC and AgRP neurons were classically considered to mediate opposite effects on food intake and energy homeostasis (Elmquist et al., 1999), with AgRP neurons stimulating feeding and inhibiting POMC neurons, the satiety neurons (Figure 1-8A). As subsequent studies have refined our understanding of these neurons, it has become clear that subpopulations of these neurons exist, which may control different functions. Many studies have sought to explore the role of disrupting expression of single proteins expressed by POMC or AgRP neurons, or more recently, activating subpopulations opto- or chemogenetically based on the projections of these neurons. Far fewer studies have examined how subpopulations are formed based on responses to different hormones or nutrients.

A series of studies examined POMC neurons (Sohn and Williams, 2012; Sohn et al., 2011; Williams et al., 2010), categorising them into subpopulations, based on their responses to leptin, insulin, and 5HT_{2C} receptor agonists. Williams et al., 2010 demonstrated that leptin and insulin receptors were mainly segregated into discrete subpopulations. These studies were extended to examine leptin and 5HT_{2C} receptors, which were none overlapping (Sohn

et al., 2011). The leptin and 5HT_{2C} receptor expressing subpopulations control different functions, as outlined in Figure 1-8B (based on (Sohn and Williams, 2012)). The overlap between insulin and 5HT_{2C} receptor expressing cells has not been examined. It is possible that there is an additional subpopulation of POMC neurons that express none of these receptors.

1.8.2 Subpopulations determined based on transcriptomics

Emerging technologies now allow the sequencing of all the RNA expressed by a cell. Three recent studies performing single cell RNA sequencing of all arcuate nucleus cells have begun to identify novel neuronal populations found in the hypothalamus, in addition to refining our understanding of known neuronal populations within the MBH (Campbell et al., 2017; Chen et al., 2017; Romanov et al., 2016). POMC neurons were divided into 3 subpopulations and AgRP/NPY neurons into 2 subpopulations, with highly expressed genes unique to one subpopulation acting as markers (Campbell et al., 2017), as shown in Figure 1-8C.

These three studies used a recently developed technique called DropSeq (Macosko et al., 2015), where cells are sorted into individual droplets, tagged with bead microparticles, which allow a unique barcode to be attached to the mRNA in the cell upon lysis within the droplet. Cells are then sequenced in pools (reducing the cost), and each mRNA can be traced back to the cell of origin based on the barcode applied. This technique allows cheap, high throughput sequencing of thousands of cells from complex tissues to be performed. However, the number of reads per cell and, as a result, number of genes detected in the original DropSeq study of the retina (Macosko et al., 2015), (and although not clearly stated, this is likely to be true in the three later studies of the hypothalamus), is lower than for a more recent single cell sequencing study of POMC neurons by Lam et al., 2017.

1.8.3 POMC Neuron subpopulations determined by transcriptomics

Lam et al., 2017 performed single cell RNA sequencing on 163 single GFP positive cells captured by FACS sorting from POMC – GFP mice. The cells were sequenced to a much higher read depth, with over 15 million reads per cell. Lam et al., 2017 identify 5 POMC neuron subpopulations, including a previously undescribed group of POMC - GFP neurons that have low POMC expression, and are AgRP/NPY positive. This observation argues against the long held view that POMC and AgRP/NPY are expressed in unique cells, and could reflect plasticity within these neurons. The subpopulations identified are outlined in Figure 1-8D. The subpopulations were defined based on statistical analyses, with some populations not

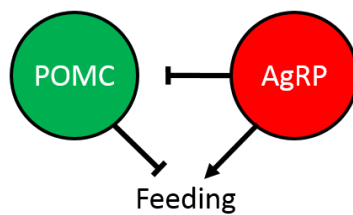
being defined by a specific marker, while others had multiple markers that were highly enriched. POMC expression varied across subpopulations. The fourth population listed in Figure 1-8D may be considered most like classical POMC neurons, with the highest POMC and CART expression, and enrichment of genes related to nutrient and hormone sensing. The authors note that statistical analysis would eventually subdivide cells back into 163 individual cells, as each cell is unique, so statistical cut-offs must be used. Notably though, the markers identified in Campbell et al., 2017 did not mark the subpopulations in this study, suggesting greater sequencing depth allows more accurate subdivision of POMC neurons.

Excluding the AgRP/NPY population (45 of 163 neurons), only 14 neurons (of the remaining 118 neurons) were LepR positive, with 6 of these neurons also expressing the 5HT_{2c} receptor, and 10 expressing the insulin receptor. 34 cells were 5HT_{2c} receptor positive, 20 of these expressed the insulin receptor. 65 POMC cells were insulin receptor positive. These data suggest that previous subpopulations identified functionally (Figure 1-8B) may not be reliable. This could be because of the lower number of neurons analysed in the previous studies (Sohn and Williams, 2012; Sohn et al., 2011; Williams et al., 2010). Another explanation is that although a gene is expressed, the gene expression level may not be sufficient to make a cell sensitive to the hormone/drug. Both sets of experiments, using slice electrophysiology or performing single cell sequencing after FACS sorting, could alter expression of genes, altering the function of the neurons. Nutritional status, age, and other factors, could also be responsible for gene expression differences between the clusters.

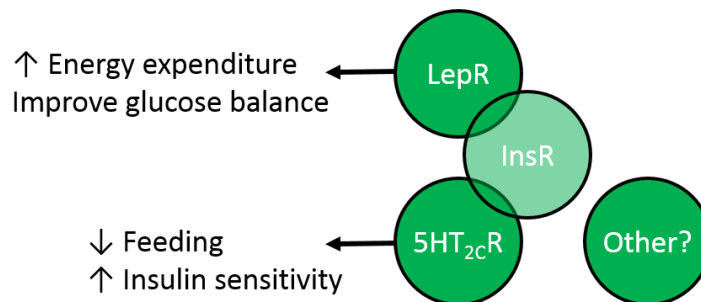
In the later study (Lam et al., 2017), some neurons in all the POMC subpopulations expressed glucokinase, suggesting glucose sensing does not drive formation of subpopulations. However, glucose sensing neurons in the MBH could be considered as a unique population that may overlap with some POMC neurons. One glucose sensing population could be those that are POMC positive.

Further work is required to confirm the existence, and function, of these POMC neuron subpopulations. In addition, how are AgRP/NPY neurons segregated? It is likely that as for POMC neurons, more AgRP/NPY subpopulations exist than those identified by Campbell et al., 2017.

A) Classical Understanding



B) Refined Understanding of POMC Neurons, electrophysiology Sohn and Williams, 2012, Sohn et al 2011, Williams et al 2010



C) Single Cell Sequencing Reveals 3 POMC, 2 AgRP Subpopulations Campbell et al 2017



D) Single Cell Sequencing Reveals 5 POMC Subpopulations No single marker drives subpopulations Lam et al 2017

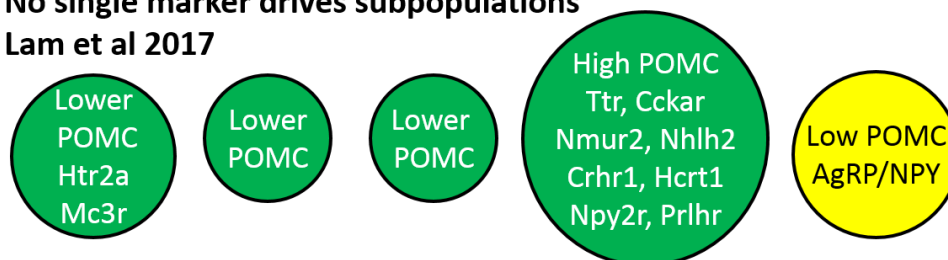


Figure 1-8: Refining the understanding of subpopulations of arcuate nucleus neurons.

Diagram outlining how subpopulations of POMC and AgRP neurons have been defined. A) Classical understanding of POMC and AgRP Neuron function and segregation. B) Studies demonstrated functional segregation of POMC neuron population, using electrophysiology and immunohistochemistry, C) RNA sequencing study identifies sub populations of POMC and AgRP neurons with unique markers for each subpopulation, D) RNA sequencing (with greater depth) reveals further subpopulations of POMC neurons, with a newly described dual POMC and AgRP/NPY positive population identified.

1.9 How is nutrient sensing organised in the MBH?

Given that in POMC neurons, nutrient and hormone sensors do not appear to drive subpopulations of neurons, how is nutrient sensing organised in the MBH? Although single cell sequencing provides useful insights, functional data of responses to stimuli is still a useful tool to understand how neurons are subdivided. As for leptin, insulin and the 5HT_{2c} receptor, studies have sought to examine how glucose and fatty acid sensing cells overlaps in the MBH. These studies provide a useful guide to generate hypotheses as to how nutrient sensing may be organised in the MBH.

1.9.1 The example of fatty acids and glucose

Hypothalamic (VMN and ARC) neurons have been shown to be sensitive to changes in concentrations of fatty acids (Le Foll et al., 2009, 2014; Wang et al., 2006); MBH fatty acid sensing can exert effects on food intake and hepatic glucose production (Le Foll et al., 2014; Obici et al., 2002; Pocai et al., 2006).

Two studies have examined the overlap between glucose and oleic acid sensing neurons in the MBH, using slice electrophysiology (on 2 – 3 week old rats) (Wang et al., 2006) or VMH hypothalamic cultures (from 3 – 4 week old rats) (Le Foll et al., 2009). Although there are some contrasting findings, which could be due to methodological differences between the studies, such as due to differing imaging techniques, use of different nutrient concentrations, and subtle age differences, several key points emerge. 1) They confirm previous findings that show that fatty acids can influence physiology by sensing in the MBH, with both studies showing oleic acid can depolarise or induce calcium responses in MBH neurons. 2) They demonstrate that glucose sensing and oleic acid sensing cells overlap, but the numbers are small, and vary between the studies. 3) They demonstrate the existence of a mechanism by which glucose regulates fatty acid sensing in neurons that may or may not be glucose sensing themselves.

The diagram in Figure 1-9A summarises the findings of these studies. The diagram is simplified, as groups for nutrient excited and inhibited neurons are not outlined. Inclusion of these neurons creates a far larger number of groups, with neurons not just excited by both nutrients, but some neurons excited by one nutrient and inhibited by the other nutrient (Le Foll et al., 2009). The bottom two groups require further clarification, but demonstrate that the interaction between nutrient sensing neurons is complex. The nutrient sensing system in

the brain is not as simple as whether a neuron responds or not to a nutrient, but depends as well on the baseline energy status of the neuron (Murphy et al., 2009a; Venner et al., 2011).

Categories of glucose and fatty acid sensing neurons identified in the MBH
Wang et al 2006, Le Foll et al 2009

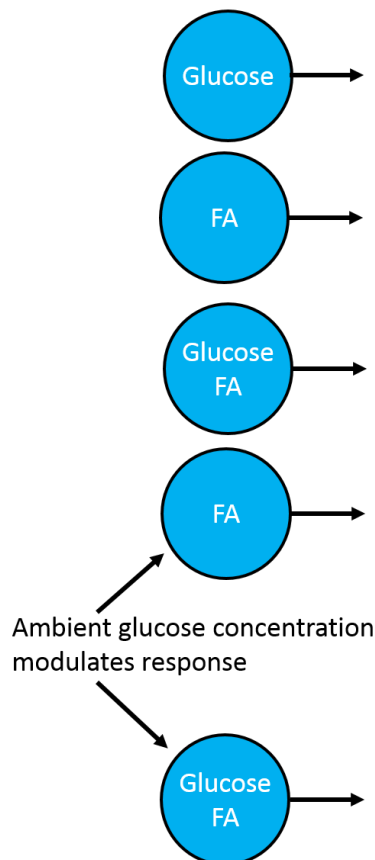


Figure 1-9: Fatty acid and glucose sensing as a model for nutrient sensing organisation in the MBH

Wang et al 2006 and Le Foll et al 2009 demonstrate the existence of multiple groups of fatty acid and glucose sensing neurons. Additional groups exist, for excited and inhibited neurons, which are omitted for simplicity.

1.9.2 Hypotheses for how nutrient sensing is organised in the MBH

Several questions emerge from these studies, regarding the organisation of nutrient sensing in the MBH:

1) How is nutrient sensing linked to the control of downstream functions? Do individual nutrient sensing cells project to single targets to control single functions (Figure 1-10A), or do nutrient sensing cells project to multiple targets to control multiple functions (Figure 1-10B), or is it a mixture of both (Figure 1-10C). Related to this question is the answer of how nutritional information is integrated (question 2).

2) How does integration of nutritional information occur: at the cellular level, or at the level of downstream circuits? Do cells that sense all three nutrients, that could serve as “metabolic sensors”, exist, as has been hypothesised (Levin, 2006; Routh, 2002)? There is evidence to suggest that integration occurs at the level of the MBH, as glucose and oleic acid sensing cells overlap in the MBH, and glucose can modulate oleic acid sensing in non-glucose sensing cells. Additionally, fasting (Murphy et al., 2009a) can enhance the glucose sensitivity of MBH neurons, and hormones can be sensed by nutrient sensing MBH neurons (Canabal et al., 2007a, 2007b; Kang et al., 2004; Kohno et al., 2003).

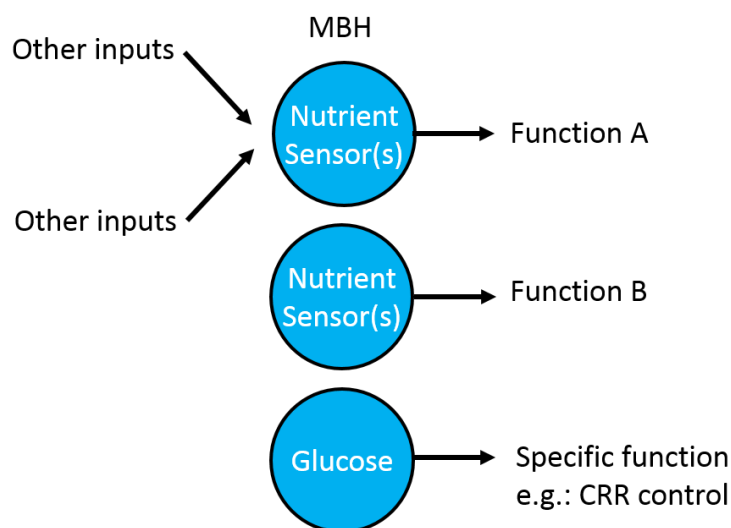
A recent study has identified that arcuate nucleus neurons such as POMC and AgRP/NPY neurons, sum synaptic inputs over time, due to expression of a sodium channel, $Na_v1.7$ (Branco et al., 2016). In most neurons, signals from synapses decay rapidly, due to natural diffusion of ions across “leaky” neuronal membranes. Expression of $Na_v1.7$ prevents decay of synaptic inputs, allowing POMC and AgRP neurons to sum inputs over time: they act as integrators over time of synaptic information. Knocking out this ion channel in AgRP neurons causes weight loss in mice, as AgRP neurons can no longer properly integrate signals of energy deficit to drive consummatory behaviour; the converse effects occur with POMC neuron specific $Na_v1.7$ knock out. How this property of these neurons modulates nutrient sensing and helps integrate nutritional information has not been examined.

3) How does mediobasal hypothalamic nutrient sensing link in to other nutrient sensing sites in the brain and body? This could be via hormones regulating MBH nutrient sensing, or through projections from other nutrient/hormone sensing sites in the brain regulating MBH neuron activity. The existence of multiple nutrient sensing sites in the brain argues for a model in which signals may converge at one point, possibly the hindbrain for some functions which generates a response via the sympathetic nervous system. For glucose, the VMN has been proposed to be a site where glucose is sensed and where other glucose sensing neurons to project to (Garfield et al., 2014; Meek et al., 2016; Sherwin, 2008). From the

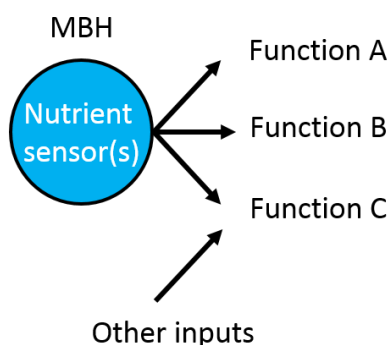
VMN, the CRR to hypoglycaemia is initiated, possibly via the hindbrain and/or BNST (Meek et al., 2016), with projections from here to peripheral sites such as the pancreas (Rosario et al., 2016).

The models in Figure 1-10 could be extended to other hormones, and other markers, such as neuropeptides, known to be involved in the control of physiology. This addresses then the much broader question, how is the mediobasal hypothalamus organised, in terms of nutrient sensing and other markers, to control physiology?

Model A: Nutrient sensing cells project to specific targets to control specific functions
Organisation at level of MBH



Model B: Nutrient sensing cells project to multiple targets to control multiple functions
Organisation at level of downstream target



Model C: Mixed model

Figure 1-10: How is nutrient sensing organised in the hypothalamus?

Models for how nutrient sensing may be organised in the mediobasal hypothalamus.

1.10 Purpose of thesis: understanding nutrient sensing mechanisms in the hypothalamus

As has been discussed, fundamental questions regarding nutrient sensing organisation in the mediobasal hypothalamus exist. Answering all these questions is far beyond the scope of one thesis. In my PhD, I have sought to answer questions in two areas. Although answering these questions is a small piece in the puzzle, the answers to them may provide important insights into the overall organisation of nutrient sensing in the hypothalamus and may provide the fundamental building blocks for future studies. With the application of emerging techniques, in combination with well used tools, more answers about hypothalamic nutrient sensing can be addressed.

1.10.1 Determining the mechanism(s) by which leucine is sensed in the MBH

In vivo data shows leucine can affect physiology when injected into the MBH. A reasonable hypothesis is that leucine produces similar responses in neurons as glucose and fatty acids do, to regulate neuronal excitability (Kang et al., 2004; Wang et al., 2006). However, this has not been demonstrated. Multiple signalling pathways have been implicated in MBH leucine sensing. Are these mechanisms, at the level of the isolated neuron, necessary for controlling neuronal excitation? Although several pathways have been implicated in leucine sensing, unlike for glucose, specific proteins that act as leucine sensors have not been shown to function as leucine sensors in the MBH, although leucine sensing candidates exist (such as Sestrin2, (Wolfson et al., 2016)). If a leucine sensor, or another molecular marker could be identified for leucine sensing cells, such as for glucose where glucokinase marks some glucose sensing cells, further studies using genetic tools to manipulate these cells would be possible.

Three key questions I have sought to answer in chapters 2 and 3 are:

- 1) Does leucine, as for fatty acids and glucose, generate neurophysiological responses at the single neuron level?
- 2) What is/are the mechanism(s) by which leucine is sensed in the MBH?
- 3) What is the identity of cells which sense leucine in the MBH?

Points 2 and 3 in combination will help to identify molecular markers for leucine sensing neurons that can be used to explore leucine sensing cells using Cre based technologies.

1.10.2 Determining the contribution of AgRP Neurons to hypothalamic glucose sensing

AgRP neurons are an important neuronal population involved in the control of feeding behaviour (Luquet et al., 2005; Sternson and Eiselt, 2017). Studies have shown that these neurons are glucose inhibited (Fioramonti et al., 2007; Hao et al., 2016), and that 30% express glucokinase (Stanley et al., 2013). Additionally, many studies, summarised in Chapter 4, have shown that disruption of AgRP neuron function alters glucose homeostasis. A recent chemogenetic study has examined the effect of activating these neurons on glucose homeostasis (Steculorum et al., 2016), but suggested AgRP neurons play novel roles in controlling glucose homeostasis, that did not support multiple previous studies (e.g.: (Könner et al., 2007)). 30% of AgRP neurons express glucokinase, so may sense glucose by this mechanism and regulate glucose homeostasis.

Two key questions I have sought to answer are:

- 1) Can the effects of AgRP neurons on glucose homeostasis as identified by Steculorum et al., 2016 be validated (chapter 4)?
- 2) What role do GK expressing AgRP neurons play in the control of physiology (chapter 5)?

Specific hypotheses are outlined in greater detail in chapters 4 and 5. In addition, I set out hypotheses as to how subpopulations of AgRP neurons may be formed, on the basis of downstream functions that these neurons are known to control, in an attempt to advance the understanding of AgRP subpopulations, as discussed in Section 1.8

2 Chapter 2: Characterization of leucine sensing properties of mediobasal hypothalamic neurons

2.1 Chapter Summary

Leucine is able to suppress appetite, and influences feeding behaviour at multiple levels. In this chapter, I set out to characterize the neurophysiological response of neurons to leucine, to determine the mechanisms by which leucine is sensed in the Mediobasal Hypothalamus (MBH) and to determine the identity of cells which sense leucine in the MBH, questions which have not been explored. The final two aims are further explored and built upon in chapter 3. Using an adult neuron culture protocol that I optimized at the start of my PhD, coupled with calcium imaging, I demonstrated that leucine bidirectionally regulates calcium fluxes in a heterogeneous population of neurons in the mediobasal hypothalamus, including POMC and AgRP/NPY neurons. Using pharmacological tools, I demonstrated that leucine sensing is mTOR independent, and is independent of leucine metabolism. Using BCH, a competitive leucine uptake inhibitor via LAT1, I demonstrated that leucine sensing occurs independently of LAT1. Calcium responses can be caused by calcium entry into the cytoplasm from outside the cell, or from intracellular calcium stores. Thapsigargin treatment to deplete intracellular calcium stores abolished leucine responses. In contrast, most neurons still sensed leucine when treated with inhibitors of store calcium release channels. Studies with calcium free media led to a large reduction in the magnitude of the response to leucine, suggesting extracellular calcium is the principal source of calcium used by cells.

2.2 Introduction

Dietary protein content can have profound effects on appetite. Although some reports suggest dietary protein content can be sensed in the periphery (Laeger et al., 2014), many reports have demonstrated a role for central amino acid sensing in the control of food intake (summarised in Heeley and Blouet, 2016). Given the important role the brain plays in integrating nutritional information, and in the subsequent control of consummatory behaviour, the ability of the brain to directly sense protein availability requires exploration.

2.2.1 *In vivo* studies studying MBH leucine sensing mechanisms

Leucine injection into the MBH or NTS has been consistently shown to suppress appetite (see Table 1 in Heeley and Blouet, 2016). The mechanisms by which changes in leucine concentrations alter neuronal firing, neurotransmitter release or other neurophysiological

properties of neurons are poorly described. Multiple signalling pathways have been implicated in leucine sensing, but it isn't known which of these pathways can couple changes in leucine concentration to changes in neuronal firing and/or other neuronal functions.

Leucine is able to modulate the meal sequence at multiple levels; the mechanisms involved in the MBH are outlined in Table 2-1. Leucine injection in the NTS acts to regulate the meal sequence in a similar way, although the leucine sensing pathways differ. As an example, leucine injection into the MBH and NTS reduces meal number. The mTOR pathway plays a role in the reduction of meal number in the NTS (Blouet and Schwartz, 2012), but not the MBH (Blouet et al., 2008). This suggests different leucine sensing mechanisms control different functions in different brain regions.

The entry of leucine into cells via a facilitative amino acid transporter, LAT1 could be a mechanism by which leucine is sensed, as has been demonstrated in *Drosophila* (Manière et al., 2016). Leucine is metabolised to form α – KIC in astrocytes and is exported to neurons (Yudkoff et al., 2005). MBH α – KIC injection, like leucine, has an anorectic effect; taken together this suggests leucine may be sensed via it's metabolite α – KIC or another downstream metabolite. This could be ATP, with Su et al., 2012 demonstrating that leucine metabolism is necessary for suppressing hepatic glucose production upon MBH injection of leucine, an effect that was dependent upon K_{ATP} channel activity (and mTOR independent).

Table 2-1: The role of different pathways in leucine sensing in vivo.

Feeding data from (Blouet et al., 2008, 2009), BAT data from (Burke et al., 2017) and glucose data from (Su et al., 2012)

	MBH Leucine's Effect					
Pathway	Reducing Meal Size	Reducing Meal Number	Increasing First Meal Latency	Decreasing Body weight	Suppressing HGP	Suppressing BAT Activity
mTOR	Yes	No	Unknown	Yes	No	Yes
Erk1/2	Yes	Unknown	Yes	Yes	Unknown	Unknown
Metabolism	No	Yes	No	Yes	Yes	Unknown
Approx. Timescale	Minutes - hours	Hours	Minutes	Days	Within 30 mins	Hours

2.2.2 *Ex vivo* studies testing MBH leucine sensing mechanisms

There have been few studies exploring the neurophysiological effects of leucine on neurons in the MBH. POMC neurons were shown to increase action potential firing in response to leucine application (Blouet et al., 2009), and similar findings were reported more recently (Smith et al., 2015). In the later study, POMC neuron specific ribosomal S6 protein kinase-1 (S6K1) knock out mice retained their ability to sense increases in leucine concentration, suggesting pathways downstream of mTORC1 are not necessary for leucine to depolarize neurons. 5mM leucine was used in the latter study, which is outside the physiological range of concentrations that these neurons are likely to be exposed to postprandially (Blouet et al., 2009; Glaeser et al., 1983; Wahren et al., 1976).

2.2.3 MBH neuronal populations implicated in leucine sensing

Both POMC and AgRP neurons in MBH may sense leucine (Blouet et al., 2009; Burke et al., 2017; Smith et al., 2015). As discussed, two studies have demonstrated that leucine can increase action potential firing of POMC neurons. Leucine injection into the MBH increased BAT temperature, the opposite effect seen when activating AgRP neurons using chemogenetics (Burke et al., 2017). It is reasonable to hypothesize that these orexigenic, nutrient sensing AgRP neurons are inhibited by leucine. So, leucine may act to increase BAT temperature by inhibiting AgRP neurons. Additionally, manipulation of S6K1 expression using viruses to over activate or quench pathway activity, demonstrates a role for the mTOR pathway in AgRP neurons in the regulation of BAT activity (Burke et al., 2017). Given that leucine potently activates this pathway, it provides strong evidence that AgRP neurons, or a subpopulation, may be leucine sensing. This occurs via the mTOR pathway, but other pathways may be involved.

The Morrison group have demonstrated that mice bearing a whole body deletion of BCATm (expressed in astrocytes in the CNS and in the periphery) have high brain leucine concentrations and increased preference for a low BCAA diet over a normal chow diet (Purpera et al., 2012). AgRP and NPY expression in the arcuate nucleus is reduced in these mice, suggesting altered AgRP/NPY neuron activity may play a role in mediating this effect. Given that mice can still choose a low BCAA diet, this also indicates that chronic abundance of BCAA in the brain induces changes in food choices that do not require BCAA transamination, suggesting the existence of leucine sensing mechanisms beyond leucine

metabolism. Additionally, in the GT1-7 hypothalamic cell line, leucine treatment suppresses AgRP mRNA expression (Morrison et al., 2007).

2.2.4 Studies of nutrient sensing *ex vivo*

While *in vivo* studies of nutrient sensing mechanisms have yielded valuable insights into leucine sensing mechanisms, these studies can be challenging due to the need for surgery to insert cannulae into the MBH. This is time consuming and low throughput. *Ex vivo* studies using techniques such as electrophysiology or calcium imaging have been used to test the mechanisms by which nutrients are sensed. With a higher throughput, promising candidate pathways can be identified and prioritized for more costly, confirmatory *in vivo* studies.

Electrophysiology compared to calcium imaging has a lower throughput, but could provide a large amount of information about how leucine acts on neurons. Calcium imaging has been used to perform higher throughput studies on glucose sensing mechanisms (Kang et al., 2004 for example) on cultured neurons from rats or mice, typically embryonic or early postnatal neurons. Adult neuron cultures are more challenging due to the increase in connective tissue as an animal ages, meaning dissociation of single neurons is harder (Brewer and Torricelli, 2007). This means harsher protocols must be used to dissociate the neurons which may affect their survival or ability to sense nutrients. Dissociation of neurons allows cell autonomous nutrient sensing properties to be observed, without the effects of neighbouring cells such as astrocytes, or other neurons projecting to the cell of interest, although arguably this is possible in slice electrophysiology using synaptic blockers. Culture models typically enrich the cultures for neurons using agents such as B27, a multi component serum free supplement developed by the Brewer lab which increases neuronal survival (Brewer and Torricelli, 2007).

Longer term studies on neuron cultures can be challenging, as cells in culture diverge in many ways from cells *in vivo*. Changes in the ability to sense nutrients have been observed, for instance, glucokinase expression was shown to halve after just 12 hours in culture, compared to freshly dissociated neurons (Kang et al., 2006).

2.2.5 Work in this chapter and hypotheses to test

During my rotation project in the Blouet lab and the start of my PhD, I optimized an adult neuron culture protocol based on the published literature (Brewer and Torricelli, 2007; Kang et al., 2004; O'Malley et al., 2006; Vazirani et al., 2013). Having demonstrated that these

neurons respond to leptin and glucose, I studied their leucine sensing properties using pharmacological tools, to determine if known pathways are involved in this acute neurophysiological response to leucine.

I hypothesise that:

- As for glucose and fatty acids, leucine activated and inhibited neurons will exist in the MBH
- POMC neurons will be leucine activated and AgRP/NPY neurons leucine inhibited
- The leucine sensing mechanism will be dependent on leucine's metabolism, involving K_{ATP} channel activation

2.3 Materials and Methods

2.3.1 Animals

POMC-EFGP and NPY-hrGFP mice (Jackson Labs) were housed under a standard 12 h light–dark cycle and were fed ad libitum. This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

2.3.2 Primary culture of post-weaning mediobasal hypothalamic neurons

Primary cultures of mediobasal hypothalamic neurons were prepared from 4 to 6 week old POMC-EFGP or NPY-GFP mice fasted overnight. Mice were killed by cervical dislocation; brains were extracted and rapidly placed into ice-cold extraction media. Two to three brain sections of 0.75 – 1mm were cut using a McIlwain tissue chopper, between approximately Anterior/Posterior -0.5 and -2.5mm from bregma. MBH wedges were dissected by eye, cutting a triangular wedge beginning halfway down the third ventricle, capturing the arcuate and ventromedial nuclei (but possibly small parts of other regions such as the dorsomedial nucleus). Tissue was placed in extraction media on ice. Tissue was transferred to papain (20 – 25U/ml, Worthington, Lakewood, NJ, USA) pre-heated at 37°C and digested for 30 minutes at 37°C under agitation (Thermomixer, 500rpm). After digestion, tissue extracts from 6 to 10 animals were pooled, washed in extraction media for 5 minutes, then transferred to a tube containing extraction media with 3.5U/ml DNase 1 from bovine pancreas (Sigma) using a glass Pasteur pipette with a fire polished 1.5mm opening, and triturated with 3 pipettes with decreasing diameters (prepared according to Nagy et al., 2006). Each trituration lasted

for 1 – 2 minutes, and tissue was allowed to settle for 4 minutes before collecting supernatant and resuspending tissue in extraction media. The trituration supernatant was gently loaded on top of a BSA gradient (4% BSA prepared in extraction media (pH 7.4) loaded on top of 8% BSA (pH 7.4)), spun for 5 minutes at 300rcf, and the pellet resuspended in culture media. 100µl of resuspended cells were plated on the glass portion of 35mm dishes (MatTek Corporation), (coated with polylysine (0.1mg/ml, Sigma)) using 0.5mm diameter trituration pipette, inside a cloning cylinder (8mm², Sigma). Plates were placed in incubator (37°C, 5% CO₂) for 1 hour. After one hour, an additional 2ml culture media was added and the cloning cylinder removed. 4 to 6 culture dishes were prepared on each experimental day. Each culture dish was imaged once and represented our experimental unit.

2.3.3 Calcium Imaging

Adapted from Diakogiannaki et al., 2013. Cells were loaded with 5µM Fura 2 AM dye (Life Technologies) for 30 minutes (15 minutes at 37°C, 15 minutes at room temperature), washed with saline and imaged using an inverted fluorescence microscope (Olympus IX71, Olympus, Southend on Sea, UK) with a 40× oil-immersion objective lens. GFP (to identify POMC or NPY cells) was excited at 488nm and fura-2 at 340nm and 380nm using a monochromator (Cairn Research, Faversham, UK) and a 75W xenon arc lamp, and emissions were recorded using an Orca ER camera (Hamamatsu, Welwyn Garden City, UK), a dichroic mirror and a 510 nm long pass filter. All images were collected on MetaFluor software (Molecular Devices, Wokingham, UK). The ratio of fura-2 emissions at 340 and 380 nm (340/380 ratio) was used to monitor changes in the intracellular calcium concentration. Solutions were perfused continuously at a rate of approximately 0.5 ml/min.

2.3.4 Preparation of Extraction and culture media

Concentrations of reagents added to prepare extraction and culture medium which are based on Hibernate and Neurobasal media from BrainBits.

Table 2-2: Composition, pH and temperature of extraction and culture media

Components	Culture media (mM)	Extraction media (mM)
Vitamins		
Choline chloride	0.029	0.029
D-Calcium pantothenate	0.008	0.008
Folic Acid	0.009	0.009
Niacinamide	0.033	0.033
Pyridoxal hydrochloride	0.020	0.020
Riboflavin	0.001	0.001
Thiamine hydrochloride	0.012	0.012
Vitamin B12	5.02×10^{-6}	5.02×10^{-6}
i-Inositol	0.040	0.040
Inorganic Salts		
Calcium Chloride	1.801	1.801
Ferric Nitrate	2.48×10^{-4}	2.48×10^{-4}
Magnesium Chloride	0.814	0.814
Potassium Chloride	5.333	5.333
Sodium Bicarbonate	26.190	0.88
Sodium Chloride	51.724	76
Sodium Phosphate monobasic	0.906	0.906
Zinc sulfate	6.74×10^{-4}	6.74×10^{-4}
Other Components		
HEPES	10.924	-
Phenol Red	0.022	0.0212
MOPS	-	10
Penicillin-Streptomycin (P4333, Sigma)	1x	1x
pH and Temperature	pH 7.6 at 37°C	pH 7.4 at room temp.

Osmolarity of all media was 260+/- 5mOsm, adjusted as necessary with sucrose. Media was prepared, without amino acids, glucose/pyruvate/lactate (GPL) or B27 (only B27 without Insulin was used, from Life Technologies, 50x). Media was filtered and stored at 4°C and used for up to 6 months.

On the day before cultures, extraction and culture media were prepared, by adding B27, Amino Acid solution (100x) and Glucose, pyruvate, lactate (GPL) solution (100x). Media was filtered.

Extraction media without calcium (pH 7.4 at 37°C) was used to prepare papain (PAP2, Worthington Biochemical). A 40U/ml stock was prepared. Papain was diluted to final concentration of 20U/ml in extraction media without calcium. Papain was not used for more than one week after reconstitution. If used more than one day after reconstitution, final concentration used was 25U/ml. GPL and amino acid solutions were added as for extraction media. No B27 was used.

2.3.5 Amino Acid Solution

Concentrations of amino acids chosen for extraction and culture medium were chosen based on published data of hypothalamic amino acid concentrations (Blouet et al., 2009; Choi et al., 1999, 2001; Currie et al., 1995; Karnani et al., 2011). A 100x stock was prepared (stored at -20°C) and added on the day before culture.

Table 2-3: Concentration of amino acids in adult neuron culture media and saline

Amino Acids	Final Concentration in culture and extraction media/mM
Glycine	0.020
L-Alanine	0.036
L-Arginine hydrochloride	0.028
L-Asparagine-H ₂ O	0.058
L-Cysteine	-
L-Histidine hydrochloride-H ₂ O	0.020
L-Isoleucine	0.003
L-Leucine	0.008
L-Lysine hydrochloride	0.067
L-Methionine	0.006
L-Phenylalanine	0.011
L-Proline	-
L-Serine	0.074
L-Threonine	0.055
L-Tryptophan	0.004
L-Tyrosine	0.009
L-Valine	0.004
L-Glutamine	0.100

2.3.6 Glucose, Pyruvate, Lactate solution (GPL).

A 100x stock was prepared (stored at -20°C), pH adjusted to 7.0 – 7.6, and added to media on the day before culture. Final concentration in media: Glucose – 2.5mM, Lactic Acid – 1μM, Sodium Pyruvate – 0.23mM

2.3.7 Saline for imaging experiments

Table 2-4: Composition of Saline for Calcium imaging experiments

Chemical	mM
NaCl	138
KCl	4.5
NaHCO ₃	4.2
NaH ₂ PO ₄	1.2
HEPES	10
CaCl ₂	2.6
MgCl ₂	1.2

For leucine sensing studies, calcium imaging was performed using saline with amino acids added to the same concentration as in extraction and culture media, and 2mM glucose. pH 7.4.

2.3.8 Glucose and leptin sensing studies

Studies with leptin and glucose were conducted with extraction media with high amino acid levels (as in commercially available media, using 50x MEM Essential Amino Acid Solution, ThermoFisher), culture media with only essential amino acids added (MEM Essential Amino Acid Solution, ThermoFisher, used as an 800x stock), and 2.5mM Glucose, 2% B27 minus insulin, 0.25% GlutaMax (ThermoFisher) and 1μM lactate added to all media. No amino acids were added in the saline. For leptin and glucose sensing studies, 2 experiments were run on each plate (including two 30mM KCl treatments). Glucose concentration in saline was 2.5mM unless otherwise stated.

2.3.9 Drugs Used

Thapsigargin (Sigma) and Rapamycin (Millipore) were prepared as 10000x stocks in DMSO. Ryanodine (Insight Biotechnology) and 2 – APB (Abcam) were prepared as 1000x stocks in DMSO. Tolbumatide and Diazoxide (Sigma) were prepared as 200x stocks in 1M Sodium Hydroxide, which was neutralized in final solution using an equivalent volume of 1M HCl.

BCH (2-Amino-2-norbornanecarboxylic acid, Sigma) was prepared in the saline solution used for imaging. Leucine, KIC and Valine were prepared as 100x stocks in water.

2.3.10 Data Analysis

Treatments were randomly assigned to each culture dish and relevant control conditions were systematically included on each experimental day. For each culture dish (experimental unit), imaging data from all cells within the 40x objective visual field were analyzed using standardized criteria for all imaging experiments, allowing unbiased analysis. Only cells showing a calcium response to KCl (max Fura2 340/380 ratio during KCl treatment at least 50% higher than baseline Fura2 340/380 ratio) and with a Fura2 340/380 ratio stable throughout baseline and washout periods (average Fura2 340/380 ratio during washout maximum 15% higher or lower than average Fura2 340/380 ratio at baseline) were included in the analysis. Cells were considered activated if showing a reversible increase in Fura2 340/380 ratio during the treatment period, with the area under the curve (AUC) of the Fura2 340/380 ratio during the treatment period at least 10% higher than during both vehicle treatment periods. Cells were considered inhibited if showing a reversible decrease in Fura2 340/380 ratio during the treatment period, with the AUC of the Fura2 340/380 ratio during the treatment period at least 10% lower than during both vehicle treatment periods. Traces were visually inspected to confirm the validity of the analysis. All statistics and graphs prepared using GraphPad Prism 6 and 7.

2.3.11 Assistance with studies.

Mouse genotyping was performed by Tamana Darwish (Research Assistant). Media preparation, on some occasions, was carried out by Tamana Darwish and Marion Arnaud (6 month visiting student who I supervised). Marion Arnaud assisted with optimization of the culture protocol, and performed immunostaining on cultures to confirm their neuronal identity. Clemence Blouet performed data analysis, as discussed later.

2.4 Results

2.4.1 Developing Adult Neuron Culture Model

During my rotation project in the Blouet lab, and through the first few months of my PhD, I optimised a protocol, based on published studies, to allow dissociated cultures of mediobasal hypothalamic neurons to be prepared. The protocol is outlined in Figure 2-1 and key optimisations performed are listed in Table 2-5. I initially worked on cultures from 6 – 10

week old mice, but moved to 4 – 6 week old mice when calcium imaging studies began to further increase the yield. Although these animals are young, they are post weaning, a period when hormone sensing properties can change dramatically (Baquero et al., 2014). As expected (Brewer and Torricelli, 2007), cells in culture were predominantly neuronal and expressed MAP2 and NeuN (data not shown - see chapter 3 for further exploration of the cell types in the cultures).

Commercially available neuronal culture media contain supra-physiological concentrations of glucose and amino acids, preventing the study of how physiological changes in nutrient concentrations are sensed by neurons. I produced my own extraction and culture media with physiological nutrient concentrations based on concentrations measured in rat cerebrospinal fluid and hypothalamic microdialysis extracts (Blouet et al., 2009; Choi et al., 2001; Currie et al., 1995; Karnani et al., 2011). These media are based on commercially available Hibernate and Neurobasal medium (Brewer and Torricelli, 2007). These media did not affect yield and viability (assessed by trypan blue staining on neurons up to 3 days in culture) compared to cultures prepared with Hibernate A (BrainBits) and Neurobasal A (Invitrogen) (data not shown), with the equivalent glucose concentrations in.

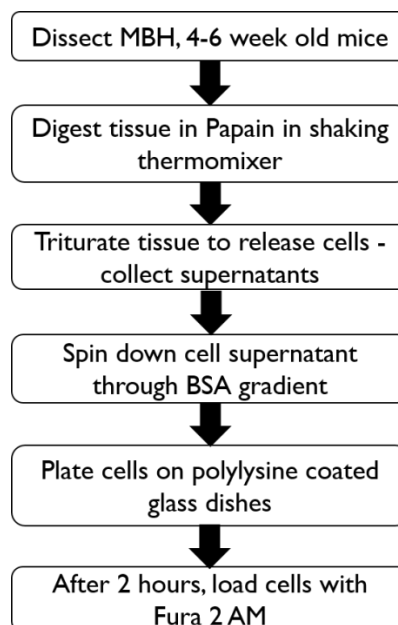


Figure 2-1: Outline of steps in adult neuron culture protocol

Table 2-5: Changes to optimize adult neuron culture protocol

Optimisation	Yield	Survival	Debris	Outcome/Comments
Age of animals	↓	-	-	Older animals lead to lower yield
Papain type (using PAP2)	↑	NE	-	PAP2 increases yield vs PAPL (PAPL contains no activators, and is cheaper)
Thermomixer for digestion	↑	NE	-	Thermomixer permits higher yield vs shaking waterbath
Tissue Dissection after digestion	↑	-	-	Small yield increase when dissecting tissue from digested brain slices, but not compatible with use of thermomixer, and reduces accuracy of dissection
BSA Gradient vs OptiPrep	↓↓	-	-	Opti Prep is not viable to remove debris where total starting number of cells is low
Layered BSA gradient	↓	-	↓↓	4% layered on 8% more effective than 4% alone
Low AA Culture Media	-	NE	-	Low AA Media does not reduce survival

NE = No effect, - = not assessed

2.4.2 Validation Studies with Glucose and Leptin

In order to assess the success of the culture model, I performed validation studies using leptin and glucose, stimuli mediobasal hypothalamic neurons are known to respond to in culture (Irani et al., 2008; Kang et al., 2004). Changes in neuronal activity were assessed by loading the neurons with the ratiometric, calcium sensitive dye, fura – 2 – AM. All traces are presented with the 340/380 fura ratio on the y axis. Neuronal responsiveness to stimuli was assessed as outlined in the Methods. All experiments ended with KCl treatment, a standard treatment that can be used in calcium imaging experiments to demonstrate cells are viable and electrically active (Pais et al., 2016a, 2016b).

A transition from 2.5mM to 5mM glucose produced an increase in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in 25% of the neurons in culture, and a decrease in $[Ca^{2+}]_i$ activated in 4.75% of the neurons in culture (Figure 2-2 A), largely consistent with previous observations (Kang et al., 2004), but with fewer glucose inhibited neurons. 10nM Leptin increased $[Ca^{2+}]_i$ in 15% of the neurons, and decreased $[Ca^{2+}]_i$ in 6.7% of the neurons, consistent with previous reports (Figure 2-2 D) (Irani et al., 2008). Examples of cells responding to glucose and leptin are shown in Figure 2-2. These data indicate that under these culture conditions,

dissociated mediobasal hypothalamic neurons from post-weaning mice retain metabolic sensing properties.

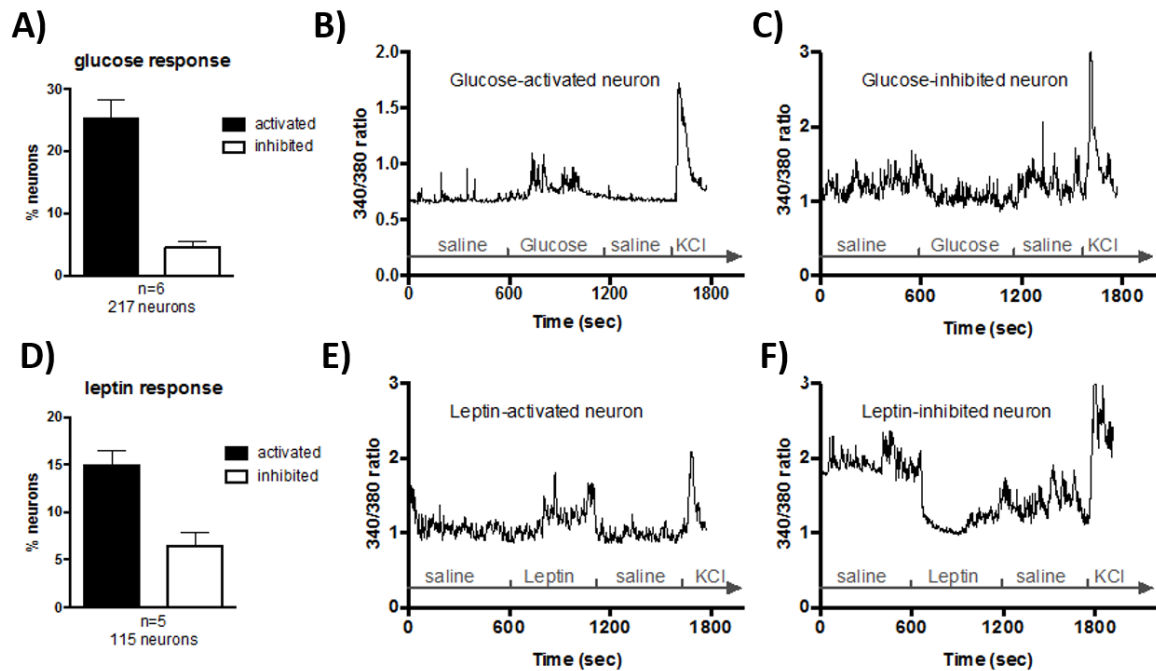


Figure 2-2: Validation studies performed with glucose and leptin.

A) % of neurons responding to an increase in glucose concentration from 2.5 to 5mM, B) Trace from a glucose excited neuron, C) Trace from a glucose inhibited neuron. D) % of neurons responding to 10nM leptin application, E) Trace from a leptin excited neuron, F) Trace from a leptin inhibited neuron. n values in A) and D) represent the number of dishes analysed, with the total number of neurons assessed written below. Bars in A) and D) are mean \pm SEM

2.4.3 A heterogeneous population of hypothalamic neurons rapidly respond to changes in leucine concentration

I used primary cultures of dissociated mouse mediobasal hypothalamic (MBH) neurons prepared from mice between 4 to 6 weeks of age to investigate cell autonomous leucine-sensing properties of mature (i.e. post-weaning) MBH neurons. I chose a concentration of 500 μ M leucine to test whether leucine caused a change in $[Ca^{2+}]_i$ in the neurons in culture. This concentration is at the higher end of the concentration range found after a high protein meal in the bloodstream (Blouet et al., 2009). Neurons in the mediobasal hypothalamus may be exposed to these concentrations after a large protein meal due to the leaky blood brain barrier in the MBH.

POMC or NPY GFP mice were used to specifically assess the changes in calcium concentration in these neuronal populations. As all neurons are loaded with fura 2, I was able to assess calcium changes in all other cells in culture. In agreement with my hypothesis (section 2.2.5), 9% of neurons were activated by leucine, and 14% were inhibited by leucine (Figure 2-3C). I also assessed the percentage of neurons that were non-specifically activated when saline was continuously applied; 2.4% of neurons were non-specifically activated, and 1.5% non-specifically inhibited.

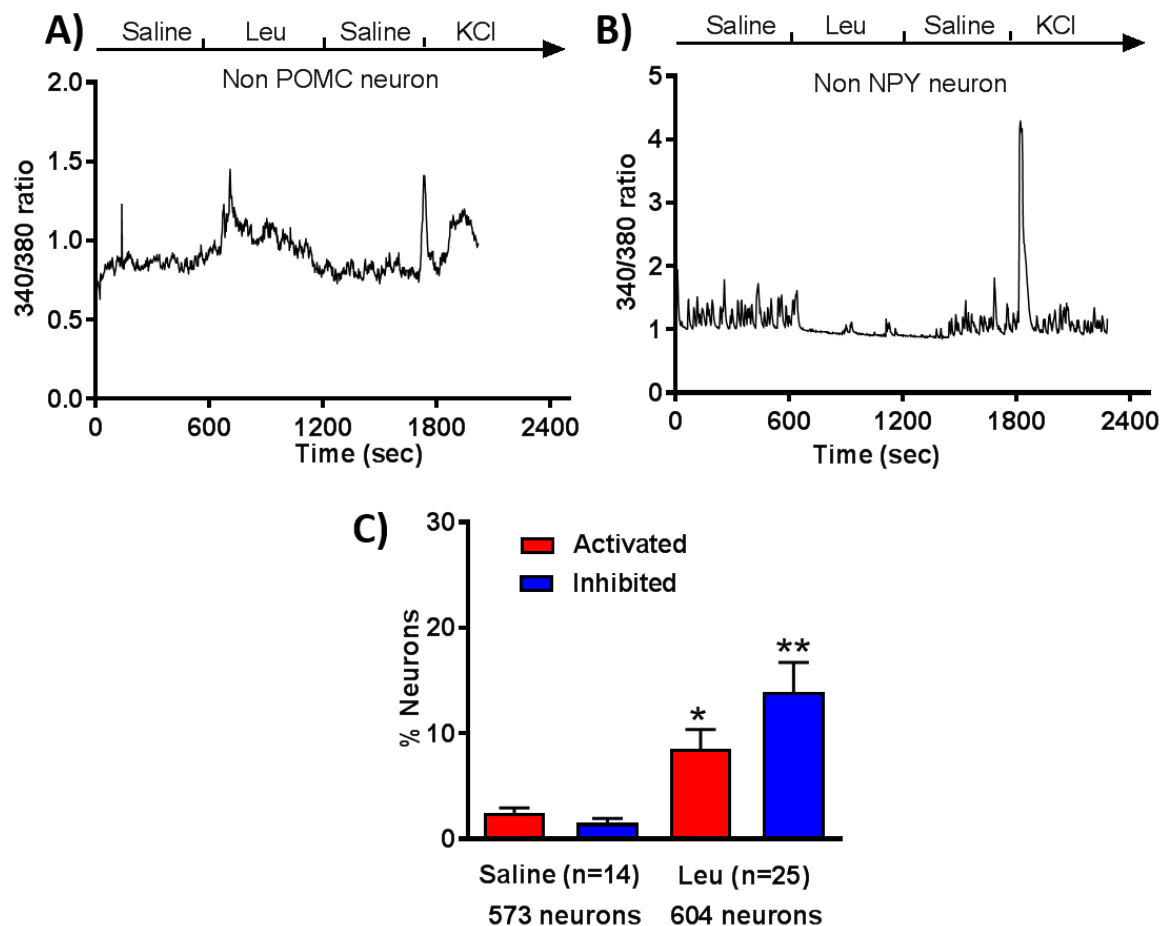


Figure 2-3: Characterisation of the response of mediobasal hypothalamic neurons to leucine
A) Trace of a leucine activated neuron, B) Trace of a leucine inhibited neuron. C) % Neurons responding. Data analysed by unpaired *t* – test. **p*<0.05, ***p*<0.01. Bars in C) are mean \pm SEM. All leucine treatments are 500 μ M.

I went on to assess the percentage of POMC and NPY neurons that responded to leucine. 21% of GFP positive cells from POMC GFP mice were activated by leucine (Figure 2-4B), while 11.9% of GFP positive cells from NPY GFP mice were inhibited by leucine (Figure 2-4D). In

contrast to my initial hypothesis (section 2.2.5), some POMC neurons (10.4%) were inhibited by leucine. Some NPY neurons were activated by leucine (5.0%), although the percentage of activated NPY neurons was not statistically significant compared to the percentage of unlabelled neurons non-specifically activated by saline. Recent data has shown that a population of POMC neurons are AgRP/NPY positive (Lam et al., 2017), so while labelled with GFP, they may have an intermediate phenotype, and so may have nutrient sensing properties of the other population.

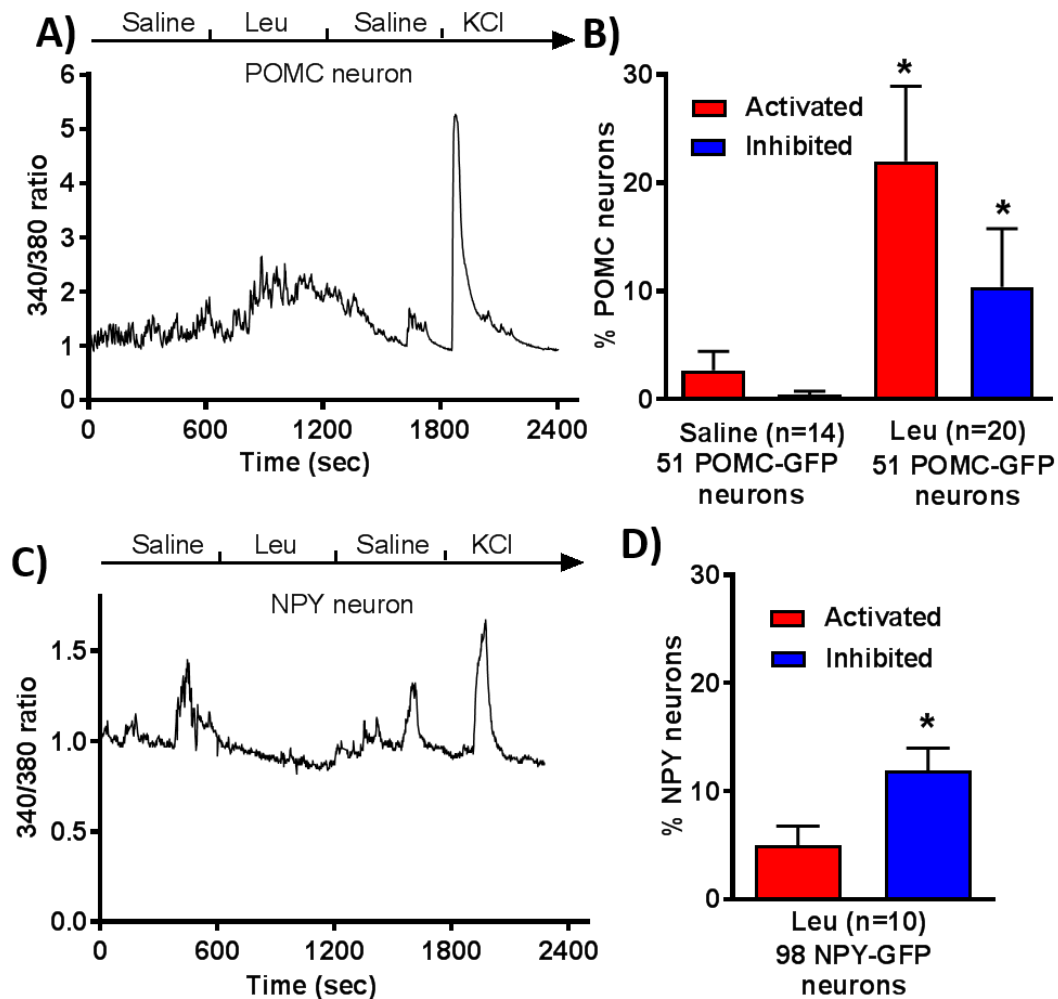


Figure 2-4: Responses of POMC and NPY neurons to leucine.

A) Trace of a POMC – GFP neuron activated by leucine and B) Percentage of POMC – GFP neurons activated or inhibited by leucine, compared to saline controls. C) Trace of NPY – GFP neuron inhibited by leucine and D) Percentage of NPY – GFP neurons activated or inhibited by leucine, when compared to the percentage of neurons non-specifically activated by saline. Data in B) and D) both analysed by unpaired t – test. $*p < 0.05$, bars in B) and D) are mean \pm SEM

2.4.4 Changes to protocol: temperature and artefacts

I initially performed experiments using an in line heater set to 37°C. However, I found that using this system, when changing solution lines, artefacts occurred when the perfusate was changed. After using various different set ups, including using infusion pumps and altering how the solutions converged before entering the bath, I finally settled on a system where imaging was performed at room temperature, using a standard gravity flow system for perfusion. Problems with artefacts still occurred on occasion. Performing imaging at room temperature may alter the response of cells to leucine if leucine is sensed by a metabolism dependent pathway.

In addition to challenges with the imaging set up, we faced challenges with data analysis. Initially I designed a spreadsheet to automatically detect cell responsive cells (10% increase or decrease in fura ratio during the treatment period). This included filtering to remove cells with high fura ratios, and irreversible KCl responses. However, Clemence and I concluded, once we began analysing data from pharmacological studies, that this method was not successfully identifying all cells that were or were not responding. We settled on a combined analysis, using a spreadsheet in combination with visually inspecting traces to confirm their response characteristics. Cells were checked against criteria listed in the Methods section to confirm responses were genuine. Clemence began the reanalysis of the data, and for consistency, continued it.

2.4.5 The rapid calcium response to leucine is independent of leucine's metabolism and K_{ATP} channels

Leucine is metabolised by BCAT to generate α – KIC. Subsequent metabolism of α – KIC to produce ATP may close K_{ATP} channels. K_{ATP} channel closure depolarises the cell membrane, leading to calcium entry into the cell through voltage gated calcium channels. α – KIC injection into the MBH mimics leucine's anorectic effect (Blouet et al., 2009), and metabolism of leucine to α – KIC in the MBH, is necessary for leucine to suppress HGP (Su et al., 2012).

Application of α – KIC activated 3.9% of neurons, and inhibited 2.4% of neurons (Figure 2-5), less than the percentage of neurons activated and inhibited by leucine (Figure 2-3C), 9% and 14% respectively, and closer instead to the non-specific activation (assessed in Figure 2-3C).

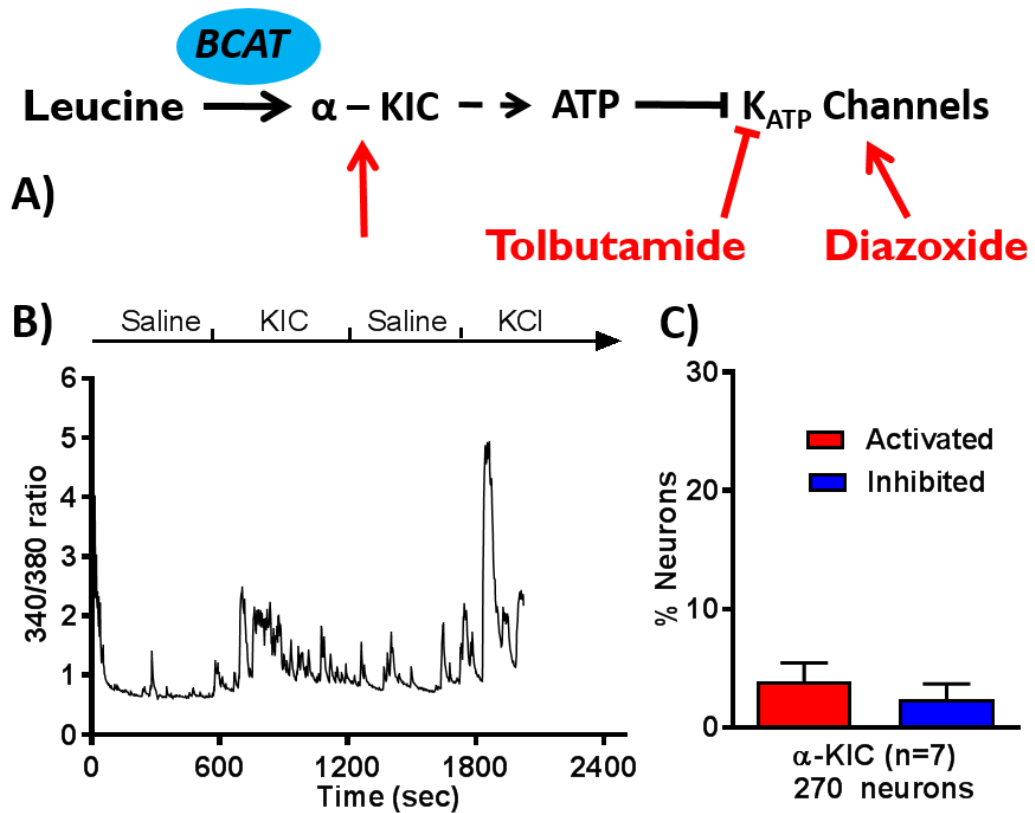


Figure 2-5: Assessing the response of neurons to the first metabolite of leucine, α-KIC.

A) Summary of pharmacological strategies to manipulate pathways related to leucine's metabolism. Dashed line indicates further metabolism. B) Trace of a neuron responding to KIC (500μM), C) Percentage of neurons activated or inhibited by α-KIC. Bars in C) are mean ±SEM

If leucine is sensed by its metabolism, then ATP produced could close K_{ATP} channels, depolarising the cell membrane leading to calcium entry into the cell. To assess if K_{ATP} channels are necessary for leucine sensing, the K_{ATP} channel opener diazoxide was co – applied during leucine treatment. A concentration of 340μM diazoxide was chosen based on published studies (Reimann and Gribble, 2002). In the presence of diazoxide, the percentage of neurons activated and inhibited by leucine was similar to the vehicle treated cells. A larger percentage of POMC cells were activated by leucine when treated with diazoxide, although the number of vehicle treated POMC cells was low, meaning conclusions are difficult to draw for POMC neurons.

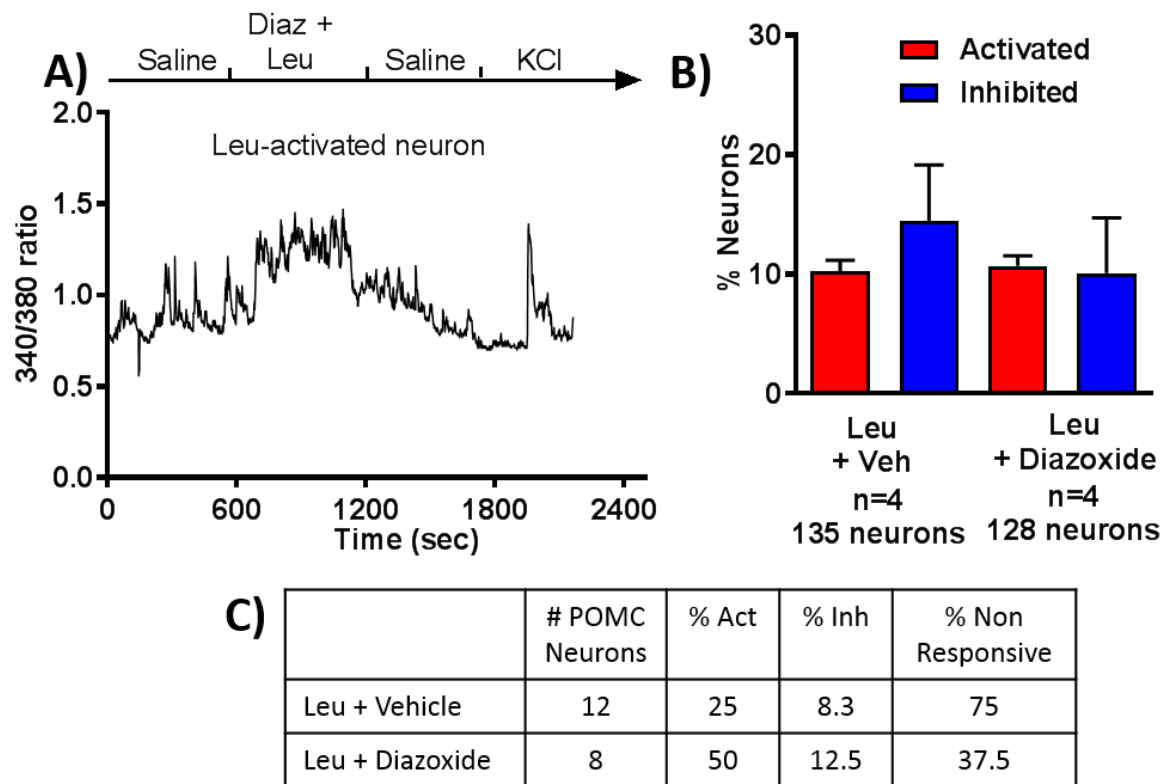


Figure 2-6: Opening K_{ATP} channels with diazoxide does not prevent leucine sensing.

A) Trace of a neuron activated by leucine in the presence of diazoxide ($340\mu\text{M}$), B) The percentage of neurons activated or inhibited by leucine in the presence of diazoxide or vehicle control, C) Responses of POMC neurons to diazoxide or vehicle treatment. Bars in B) are mean \pm SEM

However, this experimental design does not allow an assessment of the effect of diazoxide on cells, independent of leucine, to be made, so for the next manipulation, I changed the experimental design to allow the effect of tolbutamide, a K_{ATP} channel blocker, to be assessed, before seeing if leucine had an effect. A concentration of $500\mu\text{M}$ tolbutamide was chosen based on published studies (Reimann and Gribble, 2002; Reimann et al., 2008). In this paradigm, the majority of neurons that were activated by tolbutamide did not show a response to leucine. The majority of neurons that did show a response to leucine did not respond to tolbutamide, with just 2.3% of neurons responding to both tolbutamide and leucine (summarised in Figure 2-7D, and see example trace of leucine and tolbutamide sensitive cell in Figure 2-7A). I cannot exclude the possibility that some cells that respond to tolbutamide, but not leucine, are not able to respond to leucine because their K_{ATP} channels have been maximally activated by tolbutamide, suggesting these cells do use K_{ATP} channels to sense leucine. However, the percentage of leucine activated neurons (13.3%) is similar to

the percentage activated in the initial characterisation, (9% in Figure 2-3). This suggests there are not missing cells that are maximally activated by tolbutamide. POMC neuron activation does not change with tolbutamide treatment.

This data suggests that most cells that sense leucine do not express K_{ATP} channels. Therefore, leucine sensing does not require these channels. Taken with the results when testing α -KIC, it suggests leucine metabolism is not necessary for generating a rapid calcium response in mediobasal hypothalamic neurons, in contrast to my initial hypothesis (section 2.2.5).

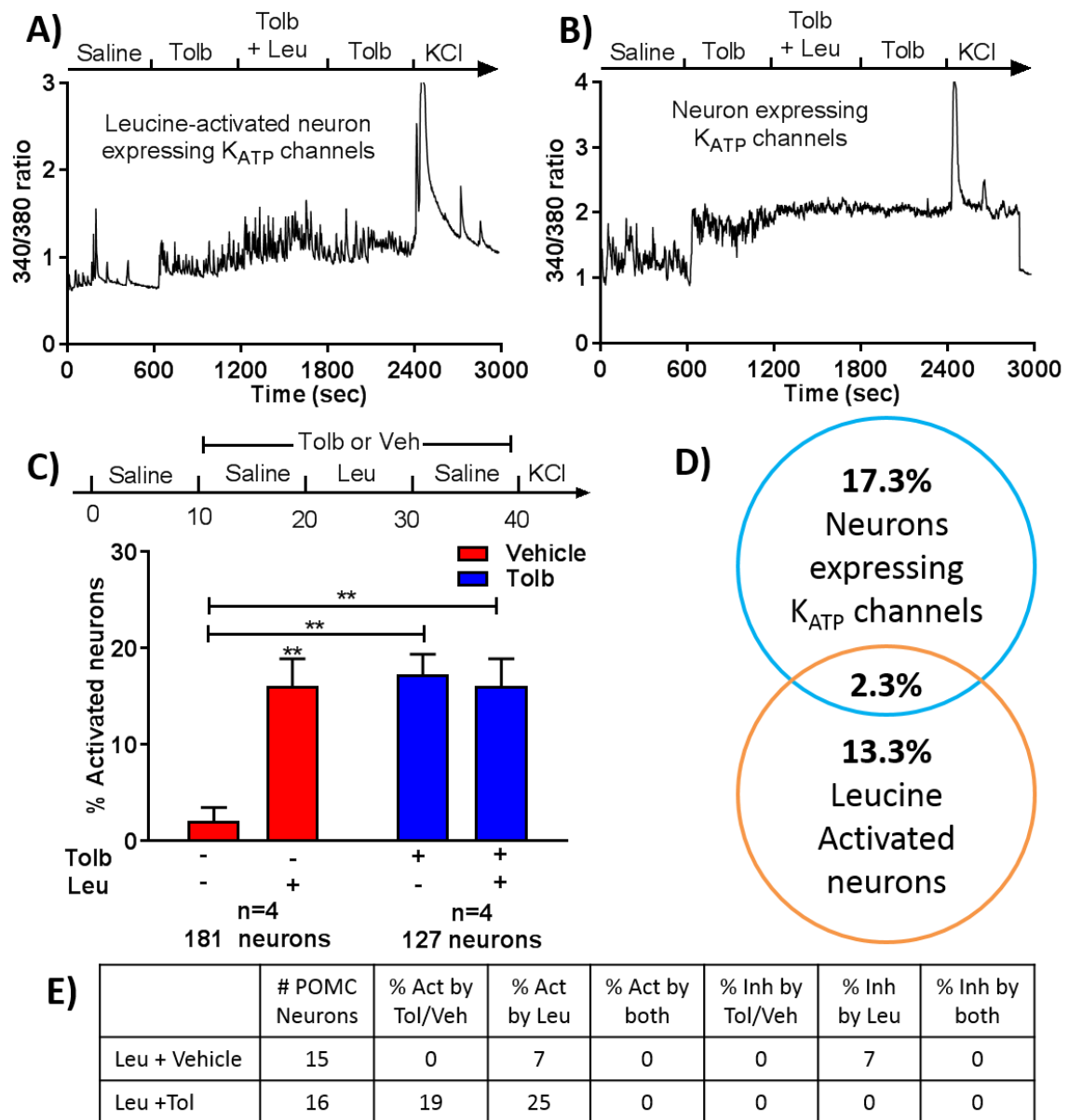


Figure 2-7: K_{ATP} channels are not expressed in most leucine sensing cells.

A) Trace from a neuron activated by tolbutamide, and by leucine, B) trace from a neuron activated by tolbutamide but not leucine, C) Graph summarising percentage of neurons activated by leucine and tolbutamide, bars are mean \pm SEM, D) Summary of the percentage of neurons activated by leucine and tolbutamide, E) Summary of responses of POMC neurons to leucine and tolbutamide. ****** $p < 0.01$, as assessed by Two Way ANOVA with Sidak's post hoc test.

2.4.6 The rapid calcium response to leucine is mTOR independent

Leucine is a potent activator of the mTORC1 pathway (Wolfson et al., 2016). Leucine's ability to be sensed by mTOR has been studied in depth in cellular systems and *in vivo*. To assess

whether the mTOR pathway is necessary for the rapid calcium response to leucine, I pre-treated cells for 1 hour with rapamycin, to block mTOR activity, based on studies in the literature (Wolfson et al., 2016), before applying leucine to cells. In cells treated with rapamycin, leucine was still able to activate and inhibit neurons (17.5 vs 15.5% cells activated, 7.8 vs 5.5% inhibited). A larger percentage of POMC cells were activated by leucine when treated with rapamycin, although the number of vehicle treated POMC cells was low.

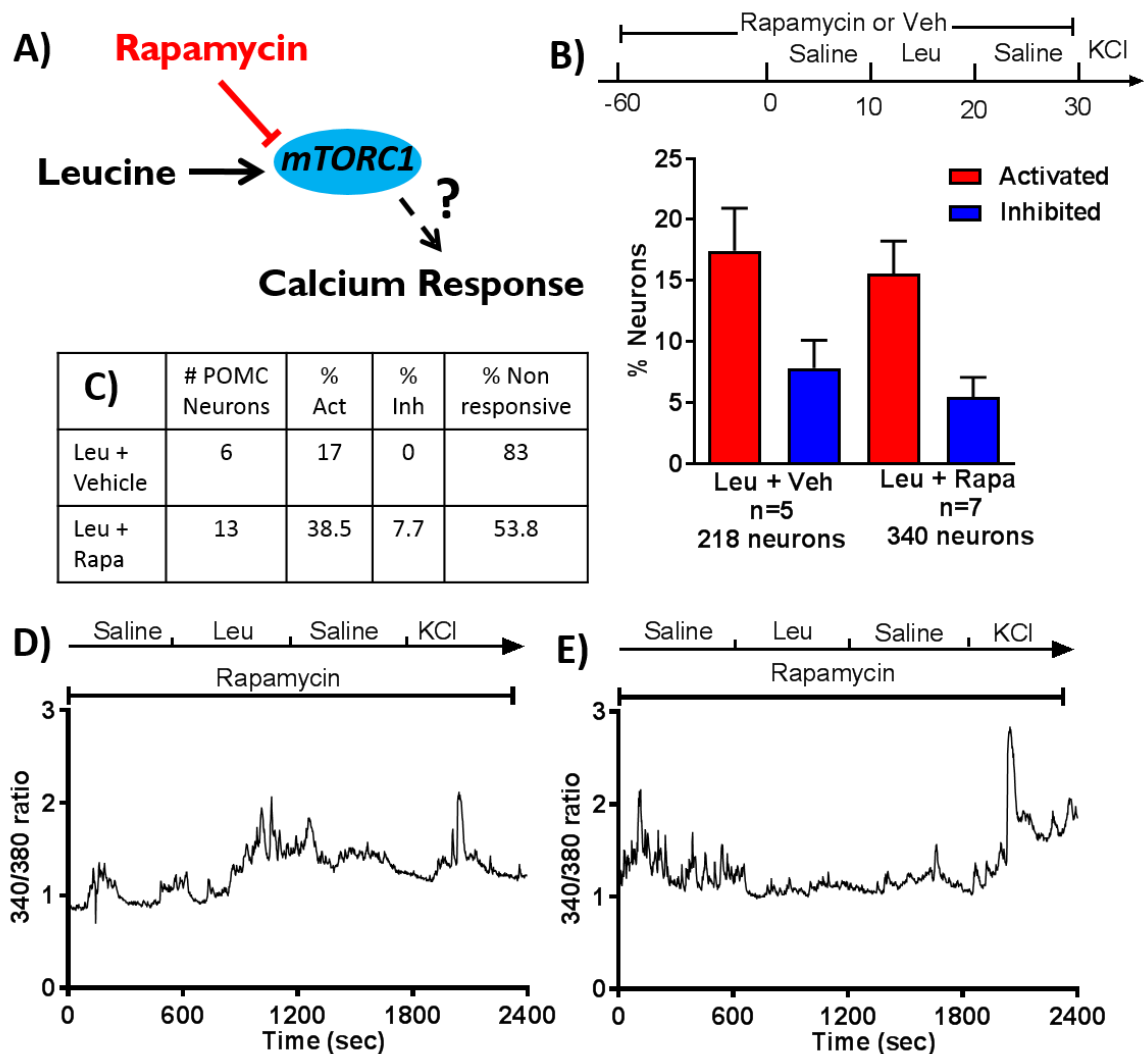


Figure 2-8: Rapamycin treatment does not block leucine responses.

A) Summary of experimental design, B) The percentage of neurons activated or inhibited by leucine, in vehicle or rapamycin treated cells, C) Summary table of the responses of POMC neurons to leucine, in vehicle or rapamycin treatment groups. D) Trace from cell activated by leucine in presence of rapamycin (100nM) and E) Trace from cell inhibited by leucine in presence of rapamycin (100nM), bars in B) are mean \pm SEM.

2.4.7 Leucine acts independently of LAT1 to initiate an intracellular calcium response

Leucine is transported into cells by amino acid transporters, principally the facilitative amino acid transporter LAT1. This may be a mechanism by which cells sense leucine abundance, as has been demonstrated for a LAT1 homologue in *Drosophila* (Manière et al., 2016). BCH is a non-metabolised leucine analogue that enters cells via the same transporter as leucine, and so can be used at higher concentrations than leucine as a competitive uptake inhibitor (Figure 2-9A)(Christensen, 1990; Kim et al., 2002; Nicklin et al., 2009; Yang et al., 2010). BCH is also an activator of GDH (Carobbio et al., 2004, 2009; Yang et al., 2010), a pathway by which leucine may be sensed, so the effects of BCH, independent of the effects of leucine, must be characterised. Figure 2-9B shows a trace from a cell activated by leucine in the presence of 10mM BCH. Figure 2-9C shows a trace of a cell activated by BCH, and also activated by leucine. The percentage of neurons activated and inhibited by leucine are summarised in Figure 2-9D – F. 13.0% of neurons are activated by leucine in the presence of BCH, as compared to 8.9% of neurons activated in vehicle treated cells. 5.6% of neurons are inhibited by leucine in the presence of BCH, compared to 4.8% of neurons in the vehicle treatment. Only 1% neurons are activated by both BCH and leucine. This data suggests leucine sensing is independent of LAT1. Given that this is the principal route through which leucine enters cells, it may suggest leucine sensing is extracellular, not intracellular.

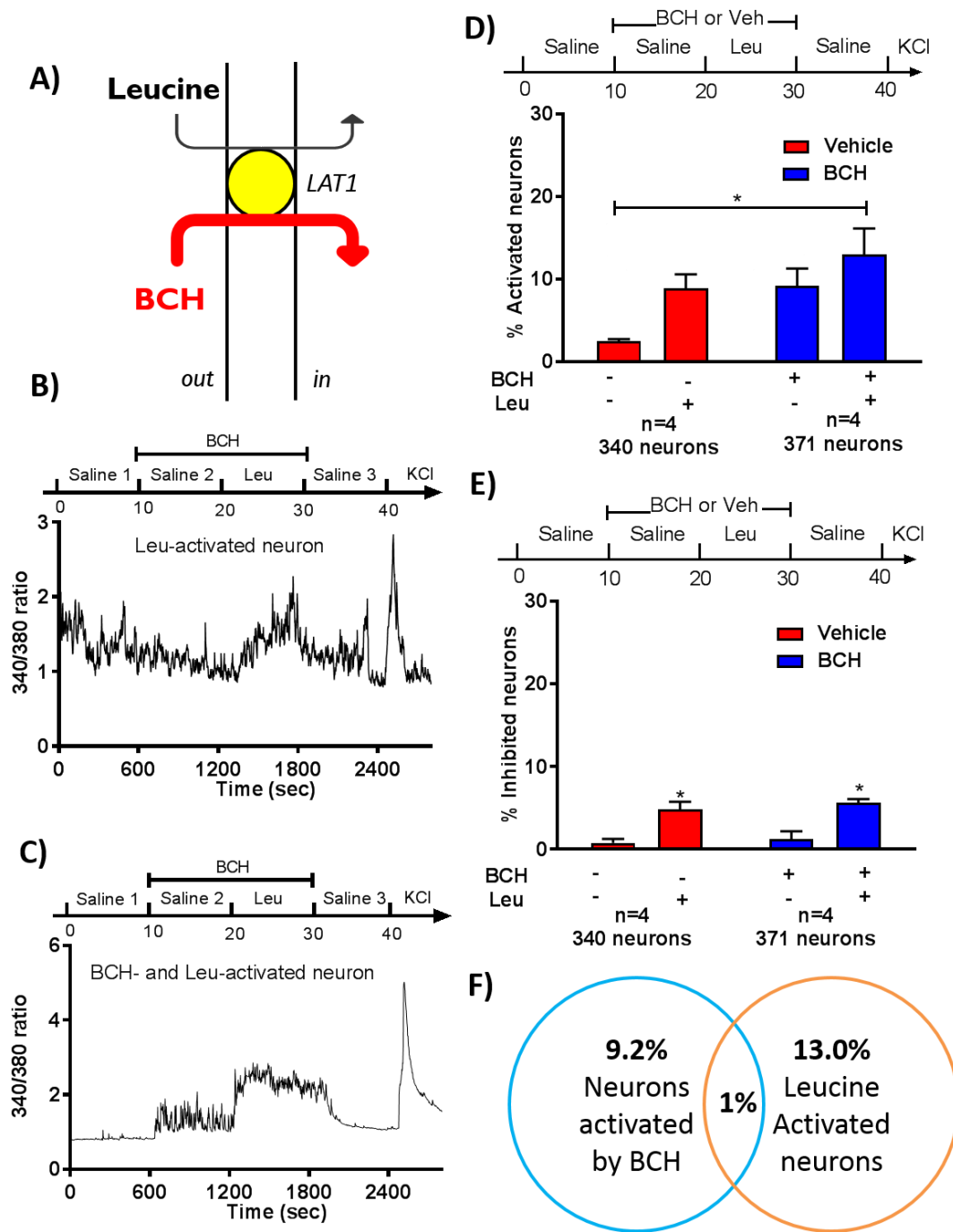


Figure 2-9: Leucine sensing is LAT1 independent

A) Summary of pharmacological strategy to block leucine uptake into cells, using 10mM BCH.

B) Trace of neuron activated by leucine in presence of BCH, **C)** Trace of neuron activated by

BCH and by leucine, **D)** Summary of percentage of neurons activated by BCH, analysed using

Two Way ANOVA with Tukey's post hoc comparison test, **E)** Summary of percentage of

neurons inhibited by BCH, analysed using Two Way ANOVA with Tukey's post hoc comparison

test, **F)** Venn diagram showing overlap between BCH and leucine activated neurons. * $p < 0.05$,

bars in **D)** and **E)** are mean \pm SEM

2.4.8 Extracellular calcium is the principal source of calcium for a leucine response

Increases in calcium concentration in the cytoplasm can be due to entry of calcium into the cell from two principal sources: entry into the cytoplasm across the cell membrane, or entry into cytoplasm from intracellular calcium stores, principally the endoplasmic reticulum, although multiple organelles may be able to contribute to cytoplasmic calcium concentration rises (reviewed by Taylor and Dale, 2012). Multiple strategies were used to interrogate what source of calcium leucine mobilises to generate an intracellular calcium response.

Initially, I tested whether the intracellular calcium stores played a role in the response to leucine. Thapsigargin is an irreversible, non-competitive inhibitor of the SERCA ATPase, that pumps calcium into the endoplasmic reticulum against its concentration gradient (Treiman et al., 1998)(Figure 2-10A). This is necessary to counteract leak currents, and necessary to refill the stores after emptying due to signalling events. Cells were pre-treated with 200nM thapsigargin for 10 min to deplete intracellular calcium stores (Treiman et al., 1998). Under these conditions, only 2% of the cells (n=4, 211 neurons) were activated by leucine (Figure 2-10C), supporting a role for calcium release from the endoplasmic reticulum in MBH leucine sensing. Some neurons were still able to respond to leucine (Figure 2-10B). Thapsigargin application is expected to transiently increase $[Ca^{2+}]_i$ as stores are depleted (Kohno et al., 2003; Muroya et al., 2004). When thapsigargin was applied, it did not cause an increase in intracellular calcium in all neurons, suggesting it may not have depleted the intracellular stores (Figure 2-10C). However, other studies have shown that thapsigargin, does not trigger a calcium response in all neurons (Kohno et al., 2011). Even without a calcium response to thapsigargin, this does not explain why thapsigargin abolishes most leucine responses.

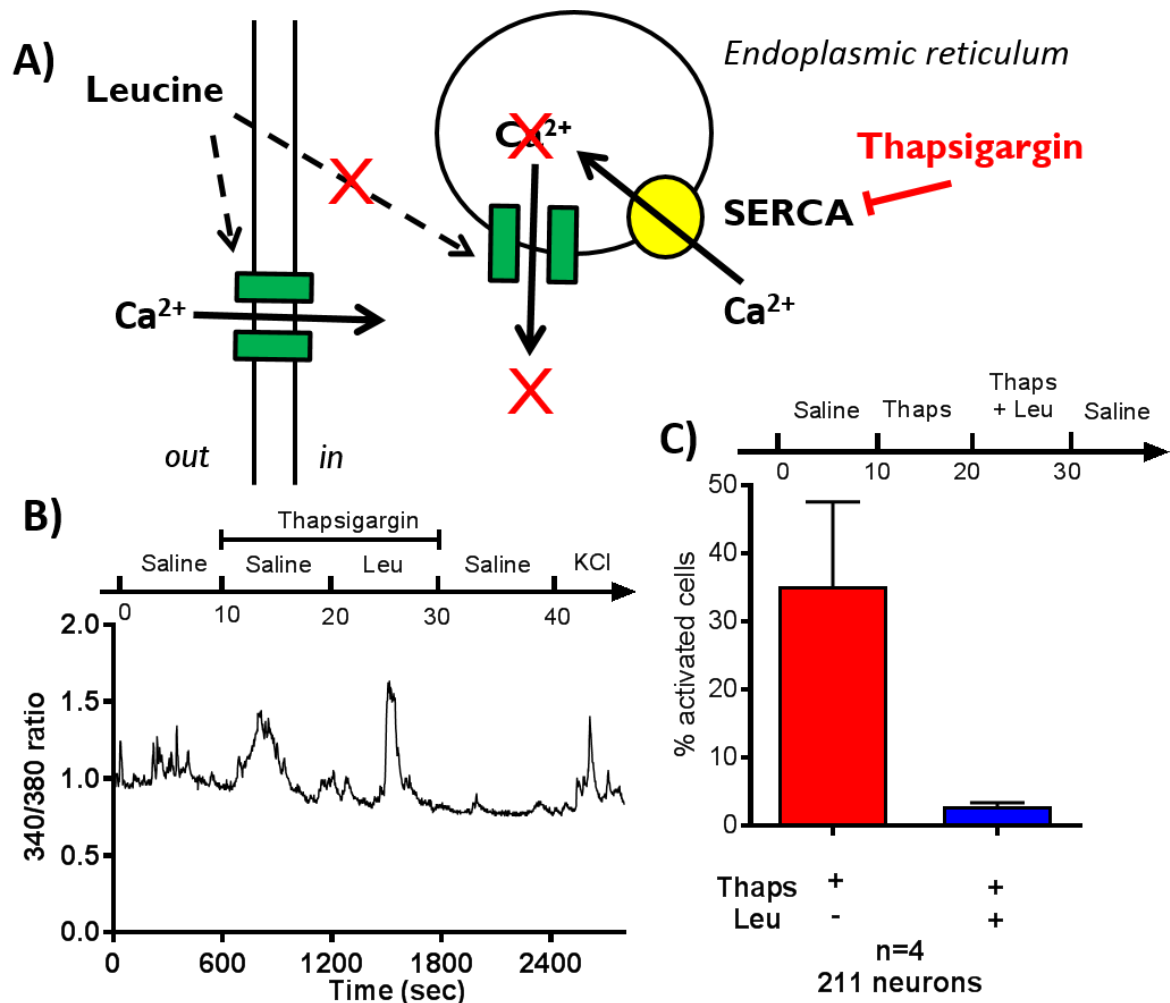


Figure 2-10: Depleting intracellular calcium stores of calcium reduces the number of neurons responding to leucine.

A) Summary of experimental design, depleting intracellular calcium stores using uptake inhibitor thapsigargin, dashed lines represent unknown links, B) Trace showing a neuron responding to leucine after store depletion by thapsigargin, C) Summary of percentage of cells activated by thapsigargin, and by leucine after store depletion by thapsigargin, bars in C) are mean \pm SEM.

I went on to examine the specific channels involved in the release of calcium from the endoplasmic reticulum. Two families of channels, IP_3 receptors (IP_3R), and Ryanodine receptors (RyR), are known to regulate release of calcium from these stores (Figure 2-11A). IP_3R 's are activated by the second messenger IP_3 , which is produced by the enzyme phospholipase C, which is activated by Gq signalling. Ryanodine receptors are typically activated by whole cell calcium waves (calcium induced calcium release) or signalling events

local to the receptor (such as IP₃R's releasing calcium) (Guo et al., 2010; Lanner et al., 2010; Thomas and Williams, 2012; Yang et al., 2011).

Above 10μM, ryanodine inhibits the opening of ryanodine receptors (Thomas and Williams, 2012; Yang et al., 2011). Ryanodine (25μM) co – application with leucine did not reduce the percentage of neurons that were activated or inhibited by leucine (Figure 2-11B – D). 2 – APB has been used as an inhibitor of IP₃R (Pais et al., 2016b; Peppiatt et al., 2003). 2 – APB (100μM) co – application with leucine did not reduce the percentage of neurons that were activated by leucine (Figure 2-11E – G). 2 – APB alone did activate some neurons (Figure 2-11E). 2 – APB did reduce the percentage of neurons inhibited by leucine, suggesting IP₃R are involved in the calcium response in some leucine inhibited neurons (Figure 2-11G).

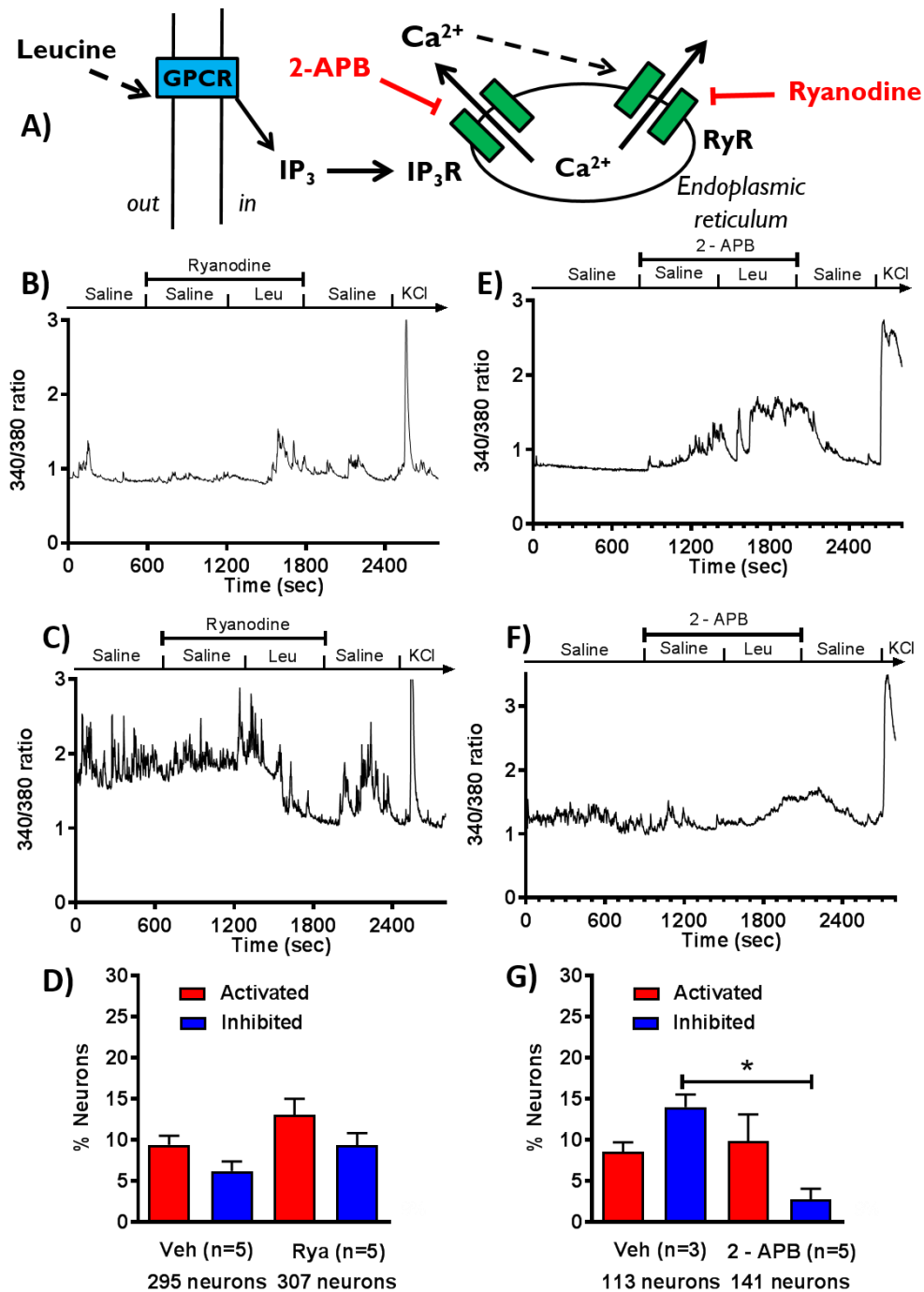


Figure 2-11: Store calcium is not the principle source of calcium for responding to leucine.

A) Summary of the pharmacological strategies to block release of calcium from intracellular stores. B) Trace of a cell activated by leucine in the presence of ryanodine (25 μM), C) Trace of a cell inhibited by leucine in the presence of ryanodine, D) summary of percentage of neurons activated or inhibited by leucine in ryanodine or vehicle treatment, E) Trace of a cell activated by leucine in the presence of 2-APB (100 μM), 2-APB also activates this cell, F) Trace of a cell activated by leucine in the presence of 2-APB, G) summary of percentage of neurons activated or inhibited by leucine in 2-APB or vehicle treatment. Results in D and G were compared using non paired t-test, $*p < 0.05$. Bars in D) and F) are mean \pm SEM.

Both ryanodine and 2 – APB did not alter the percentage of neurons activated by leucine. This is in contrast to the data found with thapsigargin, where the percentage of activated neurons was reduced when the intracellular stores were depleted. Given these contradictory results, I went on to test the contribution of extracellular calcium to the leucine response (Figure 2-12A). Neurons were incubated, after a baseline period, in media where no calcium had been added, to test whether leucine could still generate an intracellular calcium response. If so, it suggests stores are necessary for the response to leucine. Calcium removal resulted in a decrease in calcium concentrations in the cell (Figure 2-12B and C). I only pre-treated cells with this solution for 5 minutes, and then applied leucine for the standard 10 minutes, to prevent harmful effects an absence of calcium may cause (Chiesa et al., 1998; Rubin, 1970). A similar percentage of neurons were activated by leucine (Figure 2-12D) compared to the initial analysis (Figure 2-3C) but the responses were smaller in magnitude and had different response profiles and durations. This suggests extracellular calcium is a major component of the response to leucine, but may not be the initial signalling event. Of 30 POMC neurons across 6 dishes, none were activated by leucine. This suggests extracellular calcium is necessary for calcium responses to leucine in POMC neurons. It may be the initiating calcium event in POMC cells. POMC neurons may sense leucine by a different mechanism compared to non – POMC neurons.

To confirm that the calcium free conditions did not deplete intracellular stores, as some have suggested (Lu and Fivaz, 2016), I applied thapsigargin to the neurons to test if it could still mobilise calcium from the stores, which it did, in a similar percentage of neurons as seen previously (Figure 2-10C). This data suggests that extracellular calcium is a major contributor to the response to leucine, and is necessary for leucine to cause a $[Ca^{2+}]_i$ rise in POMC neurons.

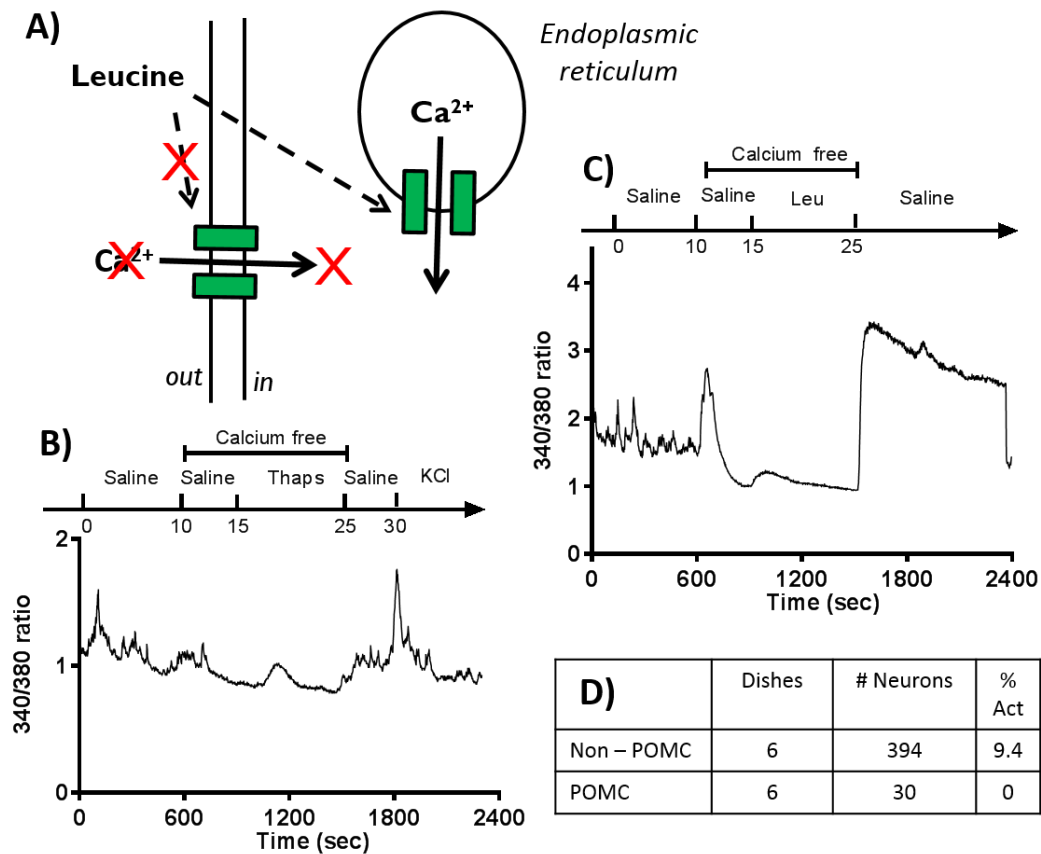


Figure 2-12: Removing extracellular calcium reduces the magnitude of response to leucine.

A) Summary of experimental design, depleting leucine from solution to test if extracellular calcium is necessary for a response, B) Positive control experiment to demonstrate that intracellular stores still contain calcium, by emptying stores to generate a calcium response using 200nM thapsigargin in calcium free conditions, C) Trace of a cell responding to leucine when calcium is absent from the extracellular solution, D) Summary of the percentage of neurons responding to leucine in the absence of extracellular calcium.

2.5 Discussion

The results in this chapter show that physiologically relevant concentrations of leucine are able to alter neuronal activity in MBH neurons. There are populations of leucine activated and inhibited neurons in the MBH, which are not only the classical feeding related POMC or AgRP/NPY neurons, although subpopulations of these neurons are leucine responsive. These responses are rapid, occurring within minutes of leucine application. These may be the neurons responsible for the rapid effects of leucine, increasing the time to eat a meal after MBH leucine injection, and for reducing first meal size (Table 2-1)(Blouet et al., 2009).

I went on to characterise the leucine sensing mechanisms causing this rapid calcium response. Most surprisingly, both the mTOR pathway, and the metabolism of leucine, were not necessary for leucine to be sensed, in contrast to my initial hypothesis. This suggests firstly, that there may be a novel leucine sensing mechanism in these MBH neurons and secondly, that leucine's effects on feeding behaviour may not just rely on changes in neuronal activity. Instead, leucine sensing through mTOR and metabolism may act in different ways in the cell to alter the activity of downstream neural circuits.

The rapamycin data is consistent with a recent study examining the effect of knocking out S6K1 from POMC neurons (Smith et al., 2015). The neurons from the POMC S6K1 deleted mice had "a more hyperpolarized resting membrane potential and lower spike frequency than control POMC neurons while input resistance was not different", suggesting S6K causes electrophysiological defects in the neurons. However, the POMC neurons from KO mice were still able to sense changes in leucine concentration (application of 5mM leucine from a baseline of zero), with modest depolarisation upon leucine application. This suggests this protein, downstream of mTORC1, is not necessary for the acute response to leucine, similar to my results demonstrating inhibition of the mTORC1 pathway does not abolish the rapid calcium response to leucine.

Studies conducted in the presence of BCH, a LAT1 inhibitor, demonstrate leucine sensing occurs via a LAT1 independent mechanism. Leucine sensing by a LAT1 like protein in drosophila fruit flies has recently been implicated in the control of food intake (Manière et al., 2016). As LAT1 is the principle entry route of leucine into the cell, this data supports a role for extracellular leucine sensing as the pathway by which leucine causes a rapid calcium response, in agreement with the data showing that the intracellular mTORC1 pathway, and leucine's metabolism, converging on K_{ATP} channels, are not necessary for leucine sensing. Leucine entry into the cell through an electrogenic transporter could be coupled to changes in calcium entry (or other ions) across the cell membrane, as is seen for amino acids asparagine and glutamine in the GLUTag cell line (Gribble and Reimann, 2016; Reimann et al., 2004), which this experiment does not test for.

GPCR's coupled to Gq can cause calcium release from IP_3 or ryanodine sensitive calcium stores in intracellular compartments such as the endoplasmic reticulum. Multiple amino acids are sensed by GPCR's, including glutamate in the CNS (mGluR's)(Hartmann and

Konnerth, 2008) and a range of amino acids or peptides in the gut (Gribble and Reimann, 2016), so leucine could be sensed this way. It has been proposed that leucine can be sensed by the GPCR's TasR1 and R3 (Wauson et al., 2012), although this is only from studies in cell lines. Depletion of the endoplasmic reticulum calcium store, by inhibiting the active transport of calcium into stores using the Calcium ATPase inhibitor thapsigargin, abolished leucine responses. This suggests that leucine responses require release of calcium from the intracellular calcium stores of the endoplasmic reticulum.

The results using thapsigargin stand in contrast to the results found from experiments with ryanodine and 2 – APB. Inhibition of ryanodine receptors using ryanodine did not block the calcium response to leucine, and using 2 – APB to inhibit IP₃R only reduced the number of cells inhibited by leucine.

At the concentration I used it at, thapsigargin is believed to be highly specific in blocking the calcium uptake pump SERCA (as reviewed by Treiman et al., 1998). The integrity of the stores depends on actively retaining calcium in the ER, but if leak currents are slow, then blocking the SERCA pump will not immediately deplete the stores of calcium. However, even if thapsigargin did not fully deplete the intracellular calcium stores, or had non-specific effects, this does not explain why the leucine responses were abolished, unless thapsigargin acts via another mechanism.

Ryanodine activates ryanodine receptors below 10μM, but at 25μM is inhibitory (Thomas and Williams, 2012; Yang et al., 2011). 2 – APB can stimulate release of calcium from stores at low concentrations, and is used as an activator, inhibitor or modulator of multiple classes of TRP membrane channels (Bellono et al., 2017). Some have questioned whether it can actually block IP₃R signalling (Bootman et al., 2002). Insufficient blockade of signalling by the receptors, by these drugs, may explain why the leucine response is not abrogated. Using a positive control that activates GPCR's, causing release of calcium from intracellular stores would allow the effectiveness of the drug concentrations to be assessed.

Finally, incubating cells in calcium free media did not reduce the percentage of cells activated by leucine, but appeared to reduce the magnitude of the response. The response of POMC neurons to leucine was abolished, suggesting they rely on calcium entry into the cell to generate a calcium response. It has been proposed that rapid buffering of intracellular calcium stores occurs in neurons, when calcium outside the cell is depleted, causing the

intracellular stores to be rapidly depleted as well (Lu and Fivaz, 2016). Thapsigargin application was still able to evoke a response in these conditions, suggesting the intracellular stores are intact (Figure 2-12B). However, we did not compare the magnitude of the thapsigargin calcium response with or without calcium present in the media, to see if some store depletion had occurred.

Taken together, the data suggests the leucine response relies on calcium release from the endoplasmic reticulum, but the principle source of calcium is from outside the cell, as outlined in Figure 2-13.

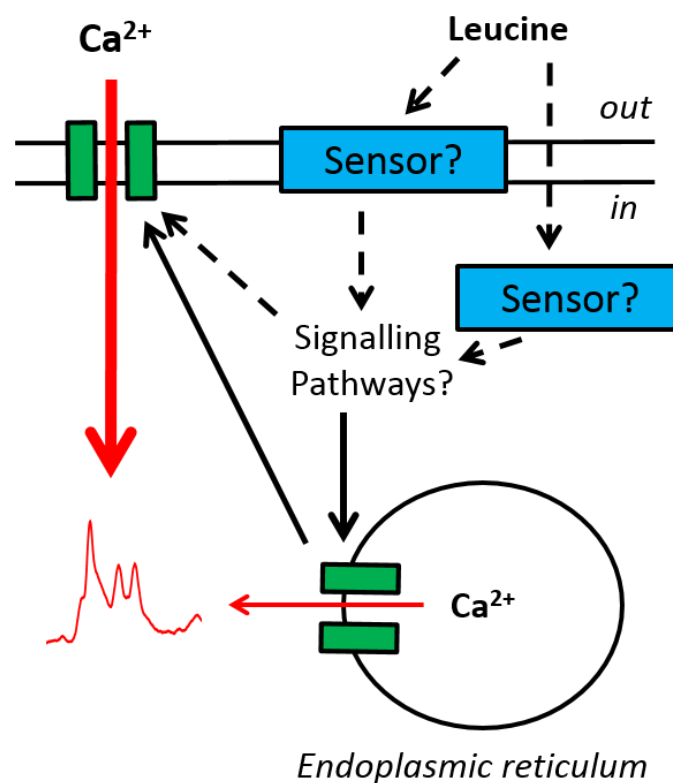


Figure 2-13: Proposed model of leucine induced calcium response in hypothalamic neurons. Dashed lines indicate unknown links. Thickness of red lines indicates proportion of calcium these routes contribute to the leucine response.

2.5.1 Leucine is sensed by multiple mechanisms

The data suggest that the rapid leucine sensing by neurons demonstrated here may occur by multiple mechanisms. POMC neurons lose their ability to sense leucine when calcium is depleted from the extracellular solution, while other neurons do still respond to leucine, albeit with a reduced response magnitude. Nearly all responses to leucine were blocked by thapsigargin, but only some leucine inhibited neurons require IP₃R to respond to leucine.

Multiple leucine sensors may exist in the cell, or multiple signalling pathways may be engaged by one sensor.

These mechanisms are in addition to other leucine sensing mechanisms such as mTOR and the metabolism of leucine previously shown *in vivo* and *in vitro* to be leucine sensing pathways (Blouet et al., 2009; Su et al., 2012; Wolfson et al., 2016). These signalling pathways are not necessary for the rapid calcium response to leucine. The Erk signalling pathway has been implicated in leucine sensing (Blouet and Schwartz, 2012; Blouet et al., 2009), but I did not test if this pathway was involved in the rapid calcium response to leucine. The data suggests that leucine is sensed by a mechanism independent of LAT1, mTORC1, and leucine metabolism, to generate an intracellular calcium response.

2.5.2 Other functions for known leucine sensing pathways

Leucine in the MBH exerts multiple effects in the control of energy homeostasis. Known leucine sensing pathways that I have shown are not involved in the acute calcium response to leucine application, such as mTOR and sensing via metabolism, may play other roles in controlling the response to leucine. These may act over different timescales to the rapid sensing mechanism described here.

Other pathways could be necessary for controlling synaptic release of specific neurotransmitters, changing synaptic strength, changing neuronal activity at the level of transcription or translation, affecting gene expression of voltage gated ion channels or synaptic proteins, such as those necessary for propagation of electrical signals within or between neurons. mTORC1, for instance regulates S6K1, which exerts control over translation via downstream factors (Cota et al., 2008; Smith et al., 2015).

The suppression of first meal latency and first meal size by leucine occurs within minutes of leucine injection in the MBH (Blouet et al., 2009). This effect could be mediated by the rapid sensing mechanism observed here. Blocking Erk signaling stopped this effect *in vivo*, suggesting the rapid calcium response could be mediated by Erk. In the NTS, the suppression of first meal latency by leucine did not occur when mTOR or Erk signaling was blocked. This raises the possibility that leucine sensing occurs via different mechanisms in the MBH and NTS.

2.5.3 Further experiments

Other methods, besides depletion of extracellular calcium, can be used to test the contribution of extracellular calcium to a stimulus response. For example, cobalt or cadmium chloride can be applied to cells, which are blockers of all voltage gated calcium channels. These can be co – applied with leucine to test whether they abolish the leucine response, implicating voltage gated channels in the response (Pais et al., 2016b). However, these heavy metal ions quench fura signals, so must be tested using a different calcium indicator, such as GCaMP. Our lab has crossed the POMC – Cre mice to GCaMP floxed mice, to enable us to test if leucine responses in POMC neurons are dependent on voltage gated calcium channels. Erk signalling could be blocked pharmacologically to test if it is necessary for leucine to cause the rapid calcium response.

Recent data suggests low leucine concentrations may be detected in the MBH via GCN2 pathway, mediating aversive responses to leucine devoid diets (Maurin et al., 2014). Leucinol could be tested, to see if this amino alcohol alters the responses of hypothalamic neurons to leucine. In addition, saline with slightly higher amino acid concentrations could be used, with the same solution minus leucine added to cells to see if MBH neurons respond to an absence of leucine.

3 Chapter 3: Identifying a molecular marker for leucine sensing cells

3.1 Chapter Summary

A molecular marker for leucine sensing cells does not currently exist. A marker would allow manipulation of these cells *in vivo* using Cre dependent technologies such as optogenetics or chemogenetics, circuit mapping tools, or knock out/knock in approaches. I used two techniques to attempt to identify a molecular marker for leucine sensing cells, phospho TRAP and single cell sequencing of leucine sensing neurons. Phospho TRAP allows the identification of markers or pathways enriched in stimulus treated animals, in our case, after MBH leucine injection. New candidate markers of leucine activated neurons were identified, which included cochlin, a secreted protein enriched in the MBH, that is fasting regulated, and a T Type calcium channel, *Cacna1g*. TTA – P2, a T Type calcium channel inhibitor, reduced the percentage of leucine activated and inhibited neurons and abolished leucine sensing in POMC neurons in calcium imaging experiments. Single cell sequencing was performed on cells identified as leucine sensing by calcium imaging. 12 control neurons, 11 leucine activated, and ten leucine inhibited neurons were sent for sequencing, including POMC and NPY GFP positive cells, with a 10% failure rate. Seven leucine sensing neurons were TH positive. Dopamine signalling is also a pathway enriched in pathway analysis from the phospho TRAP study. Half leucine sensing neurons were glucokinase positive, although many control cells were also GK positive, and expression of other glucose sensing markers was low or absent. This suggests there is some overlap between glucose and leucine sensing neurons in the MBH. Further cells are required to perform a more detailed analysis to identify unique markers for leucine sensing cells.

3.2 Introduction

My results from Chapter 2 suggest that leucine is sensed by a heterogeneous population of neurons in the MBH. In this chapter I used two techniques to attempt to identify a molecular marker of this heterogeneous population of leucine activated neurons. This marker may also give insights into the mechanisms of leucine sensing.

3.2.1 Identifying Novel leucine sensing mechanisms: Phospho TRAP

The principle of the Phospho TRAP study we conducted is outlined in Figure 3-1, adapted from (Knight et al., 2012). The authors observed that multiple signalling pathways converged on a series of serine phosphorylation events on ribosomal protein S6. Testing with a large

number of stimuli demonstrated there was significant colocalisation in the brain between pS6 (phosphorylated S6) and expression of cFOS, a marker of activated neurons widely used in the field (Knight et al., 2012). This demonstrates pS6 is a reliable marker of activated neurons.

Using antibodies, phosphorylated ribosomes can be immunoprecipitated. The mRNA being translated can then be quantified by qPCR or RNA sequencing. By comparing the mRNA expressed in the total tissue taken before immunoprecipitation, to the mRNA in the immunoprecipitate, an enrichment ratio can be calculated. This enrichment ratio for samples taken from stimulus treated mice is compared to control treated mice, to correct for markers found in activated neurons in baseline conditions. This allows markers enriched in stimulus activated neurons to be identified.

Additionally, markers of neurons inhibited by a stimulus can also be identified. Basal pS6 phosphorylation can be reduced in inhibited neurons, meaning a reduction in the enrichment ratio (below 1) should identify markers of inhibited neurons (Knight et al., 2012).

The technique has been used to identify molecular markers for neurons activated by a variety of stimuli, such as fasting, a salt challenge, ghrelin (Knight et al., 2012), and more recently, warm sensitive neurons in the preoptic area of the hypothalamus (Tan et al., 2016). To our knowledge no one has used the technique to test the effect of activating neurons after a discrete brain injection.

If a pathway's activity is upregulated or downregulated in leucine sensing neurons at the transcriptional level, this technique should also detect enrichment of these pathways. This is because the relative enrichment in the i.p. (immunoprecipitated) samples when gene expression increases in activated neurons will be larger than in the tissue overall (input samples).

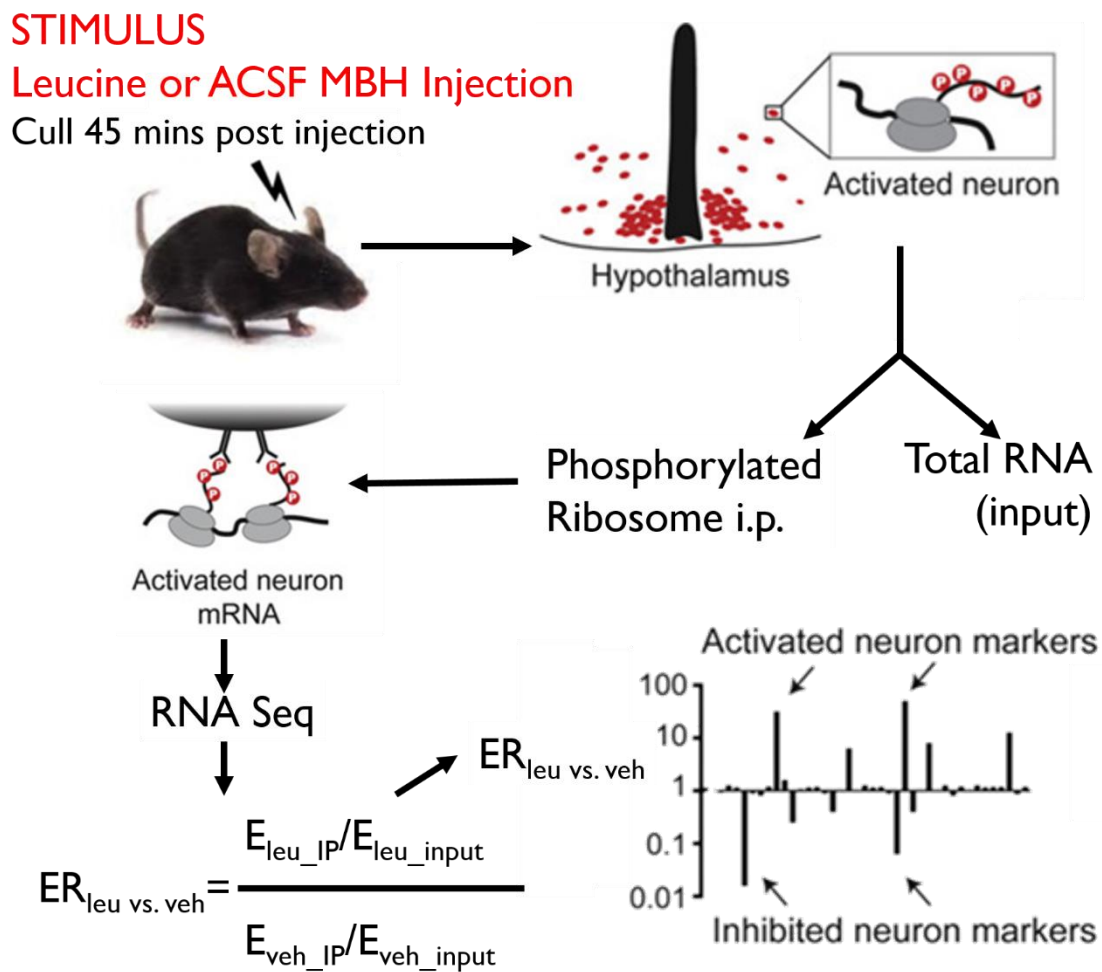


Figure 3-1: Flow diagram of steps in Phospho TRAP protocol

Figure adapted from Knight et al., 2012, with leucine injection as stimulus. We culled animals 45 minutes after leucine injection, but different time courses can be used. ER = enrichment ratio. i.p. = immunoprecipitation.

3.2.2 Identifying Novel leucine sensing mechanisms: Single Cell RNA Sequencing

In the glucose sensing field, single cell PCR was used to assess whether known proteins involved in glucose sensing, were expressed in glucose sensing neurons, identified using calcium imaging (Kang et al., 2004). This has been extended by the Levin lab to study fatty acid sensing (Le Foll et al., 2009). The extremely low amount of RNA means only a limited number of targets can be assessed. Both the low amount of RNA and lack of knowledge of targets would present a challenge if searching for novel markers of a responsive cell. Some targets may be very lowly expressed due to the proteins having a low turnover rate (Khan and Joseph, 2003; Wojcikiewicz, 2012). The culture model can alter the expression of the

sensing machinery, as demonstrated for glucokinase, with its expression halving in culture within a day of extraction, meaning it is hard to identify what mechanism is used for nutrient sensing (Kang et al., 2006). Nutrients may alter expression of the components of the signalling pathway necessary for nutrient sensing. All of these factors present challenges for these studies.

Advances in technology now allow sequencing of all RNA expressed in a cell to be conducted. Three recent studies (Campbell et al., 2017; Chen et al., 2017; Romanov et al., 2016) have performed Drop – Seq on hypothalamic cells, which has allowed the identification of new neuronal subtypes, the classification of all cells found in the hypothalamus, and sub grouping of known populations of hypothalamic neurons. These studies of tens of thousands of cells are powerful analyses that have expanded our understanding of the organisation of the hypothalamus, and heterogeneity within the hypothalamus. Lam et al., 2017 sequenced around 150 single GFP positive cells captured by FACS sorting from POMC – GFP mice, but performed much deeper sequencing on these neurons, with over 15 million reads per cell, compared to the other single cell sequencing studies. Although the DropSeq papers do not state their read depth, the number of cells sequenced means the read depth is likely to be far lower. This is seen in the number of sub groups of POMC neurons identified. Campbell et al., 2017 identify 3 subgroups of POMC neurons, whereas Lam et al., 2017 identify 5 subgroups, including a previously undescribed group of POMC - GFP neurons that are AgRP/NPY positive. Looking at a larger number of neurons means the confidence in each subgroup identified is higher as more cells are seen in each group, but the lack of sequencing depth means subtle differences between subgroups are not observed. This will be a challenge going forward, with a balance to be found between sequencing depth versus number of cells versus the cost of sequencing so many cells.

However, the challenge with these studies is to translate this knowledge of organisation into an understanding of the function of the hypothalamus. Coupling single cell RNA sequencing to functional studies, such as identifying whether neurons are activated by a stimulus, can provide deeper insights into mechanisms of nutrient sensing, and the function of the hypothalamus.

3.3 Materials and Methods

3.3.1 Animals, stereotactic surgery and acclimatisation

6 – 8 week old Wild Type C57BL/6 mice (Charles River UK) underwent stereotactic surgery to allow implantation of a bilateral cannulae, allowing injection at coordinates -1.1mm Anterior/Posterior, -5.9mm Dorsal/Ventral from bregma. The cannulae tips were spaced 0.8mm apart, allowing injection 0.4mm either side of the midline, to target the MBH. Mice were handled regularly before being studied, 8 – 12 days post-surgery. All mice were housed under controlled temperatures (21–23°C) and a constant 12 hr light/dark schedule and fed ad libitum. This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

3.3.2 Study Design

On study day, mice were fasted at 11.15am. Leucine or ACSF was injected at 4.30pm, following an identical design and volume to Blouet et al., 2009. At 5.15pm animals were injected with an overdose of anaesthetic, before brains were removed onto ice. The study was performed on 4 days, with 5 mice in each treatment group on days 1 – 3, and 4 mice per treatment group on the fourth study day. Tissue was pooled from the same treatment groups on each study day, to give 4 sets of ACSF vs leucine samples.

3.3.3 Tissue Collection and preparation of input samples

Adapted from Knight et al., 2012. The mediobasal hypothalamus was dissected in a 1xHBSS buffer with, 4 mM NaHCO₃, 2.5mM HEPES (pH 7.4), 35mM Glucose and 100 mg/ml cycloheximide (cycloheximide prepared beforehand in methanol) (Buffer B). Pooled hypothalami were then manually homogenized in a 1ml of homogenization buffer (buffer C) containing 10mM HEPES (pH 7.4), 150mM KCl, 5mM MgCl₂, 100nM calyculin A, 2mM DTT, 100U/ml RNasin, 100 mg/ml cycloheximide and Roche protease and phosphatase inhibitor cocktails. Samples were kept on ice, before being transferred to a new tube for centrifugation at 2000g, 4°C for 10 minutes. The supernatant was transferred to a new tube, and 70µl of 10% NP40 and 70µl of DHPC (1,2-diheptanoyl-sn-glycero-3-phosphocholine) per 1 ml supernatant was added. Samples were mixed by inversion and incubated on ice for 2 minutes. Samples were then spun at 16100g for 10 minutes. Supernatants were transferred to a new tube. 25µl was collected into a new tube with 350µl RLT and stored at -80°C. These

were the total RNA samples, now known as input sample. The remaining sample was used for the immunoprecipitation (i.p.).

3.3.4 Bead preparation, immunoprecipitation and RNA extraction

Protein A Dynabeads (Invitrogen) were washed using wash buffer (wash buffer A) containing 10mM HEPES, 150mM KCl, 5mM MgCl₂ and 1% NP40 (pH 7.4), before being incubated with 4µl of pS6 antibody against Ser240/244 (#2215, Cell Signalling Technology) in 0.1% BSA (per 100µl beads), overnight at 4°C. 300µl of beads per i.p. were washed twice with wash buffer (wash buffer D), containing 10mM HEPES (pH 7.4), 350mM KCl, 5mM MgCl₂, 2 mM DTT, 1% NP40, 100U/ml RNasin, and 100mg/ml cycloheximide, and Roche protease and phosphatase inhibitor cocktails, using a magnet to catch the beads. Beads were resuspended with 200µl of buffer C supplemented with NP40 and DHPC as above, and 1µM ZK10 (gift from Zackary Knight).

The remaining supernatant was added to the antibody beads, resuspended in Buffer D and mixed by inversion for 10 minutes at 4°C. Samples were placed on the magnet, and washed 4 times with 0.9ml of ice cold buffer D. Beads were resuspended, then placed on magnet so supernatant could be removed. After resuspending the beads during the 3rd wash, the beads were transferred to a new microcentrifuge tube, to eliminate any RNA that may be non-specifically stuck to the tube. After transferring to the new tube, the beads were incubated in the tube at room temperature for 10 minutes before removing the supernatant from the third wash. After removing the supernatant following the fourth wash, 350µl RLT buffer was added to the beads, for 5 minutes on ice. The tubes were then placed on the magnet and supernatants were collected into a new tube. This supernatant is the i.p. RNA. The RNA was stored at -20°C. RNA was extracted using the Qiagen RNeasy Micro kit, and RNA quality and quantity assessed using Pico Chips on the Agilent Bioanalyzer, and the Ribo Green Assay (Thermo Fisher).

3.3.5 RNA Sequencing of PhosphoTRAP samples

Based on Lam et al., 2017. 1ng of RNA was used as input into the Smart Seq v4 Ultra Low Input RNA kit (ClonTech) for preparation of cDNA, according to manufacturer's instructions. 11 PCR Cycles were used for both i.p. and input samples. cDNA was generated, and 150pg in 5µl was used for input into library preparation using the Illumina Nextera XT Kit. Libraries for sequencing were prepared according to manufacturer's instructions.

The sequencing libraries were normalized to 10 nM concentration and pooled. The pooled libraries were sequenced on an Illumina HiSeq 4000 instrument at single-end 50bp (SE50). The sequencing was performed at the Genomics Core, Cancer Research UK Cambridge Institute.

3.3.6 Data Analysis for PhosphoTRAP study

Data was collected and analysed by Clemence Blouet and Brian Lam, as outlined in Lam et al., 2017. The RNA sequencing data that is produced must be aligned to the genome: the sample reads are matched to the location of the genome the RNA was transcribed from. After this alignment, the FPKM value is calculated. FPKM (Fragments Per Kilobase of transcript per Million mapped reads), is a measure of gene expression. It is calculated by measuring the number of reads of a transcript of a gene collected during a sequencing run, in proportion to the total number of reads within the whole sequencing run. It is normalised for the length of the transcript, as a longer transcript will have more total reads in the sample although it is not more highly expressed. This allows a direct comparison between transcripts of varying lengths. This process is performed using Cufflinks software.

Four sets of samples were generated. Each set contained a leucine i.p. sample ($E_{\text{leu_ip}}$), leucine input sample ($E_{\text{leu_input}}$), vehicle i.p. sample ($E_{\text{veh_ip}}$) and vehicle input sample ($E_{\text{veh_input}}$). Sequencing was performed, and an FPKM for each gene was produced for each sample. The enrichment ratio (ER leu vs veh) for each gene in each set was then calculated as stated in *Figure 3-1*. The E_{leu} was calculated by taking $E_{\text{leu_ip}}/E_{\text{leu_input}}$. E_{veh} was calculated by taking $E_{\text{veh_ip}}/E_{\text{veh_input}}$. The overall enrichment ratio (ER leu vs veh) for each gene was then calculated as follows: $E_{\text{leu}}/E_{\text{veh}}$.

P values were calculated by comparing the i.p./input ratio for leucine treatment (E_{leu}), to i.p./input ratio for vehicle treatment (E_{veh}), using Cufflinks software. Candidate genes were identified if p value was statistically significant ($p < 0.05$) and FPKM values for all the samples were > 1 . This gave a list of 478 genes, which was refined to a list of 48 genes by increasing analysis stringency ($p < 0.02$, $\text{FPKM} > 1$, enrichment ratio > 2 or < -2). This produced a list of candidate genes.

Pathway analysis was performed by Clemence Blouet using Ingenuity Pathway Analysis. Ingenuity pathway analysis is a piece of software developed by QIAGEN. It determines which known cellular pathways are up or down regulated. It allows comparison between data sets,

in this case, to determine whole pathways up or down regulated by the leucine treatment. It produces a z – score, with more positive or negative values representing pathways more strongly up or down regulated by the treatment. The software then uses statistical tests to determine if those changes are statistically significant.

3.3.7 Calcium Imaging

Calcium Imaging was performed as outlined in Chapter 2. TTA-P2 (Alomone Labs, Israel) was prepared as 10mM stock in DMSO.

3.3.8 Single Cell RNA Sequencing

After identifying cells that were activated or inhibited by leucine, or not responsive to leucine using calcium imaging, single neurons were collected into autoclaved glass pipettes, fire polished to an opening that gave small resistance when applying 0.65ml of positive pressure when attached to a sealed system, using a Micromanipulator. Cells were collected into lysis buffer (8.5µl lysis buffer per cell), according to manufacturer’s manual (Clontech Smart Seq v4 kit). RNA from the single cell lysates was processed as in Lam et al (2017), and bioinformatics performed as in this study.

3.3.9 Assistance with studies

Clemence Blouet and I performed the surgery for the Phospho TRAP studies together. Clemence performed the brain injections, and we performed the Phospho TRAP together. Marcella Ma assisted with optimisation of the RNA sequencing protocol. Brian Lam and Clemence Blouet performed the data analysis on the Phospho TRAP data set. Anthony Tsang has validated candidate hits using qPCR. Irene Cimino prepared libraries for the single cells for RNA sequencing, and Brian Lam aligned the sequencing data, providing a table of FPKM values for analysis. Gwen Tolhurst assisted with initial single cell capture experiments, and Tamana Darwish prepared the cell culture media for these experiments. We thank Professor Sadaf Farooqi for identifying Phospho TRAP hits that were also hits in the GOOS cohort.

3.4 Results

3.4.1 Confirming success of PhosphoTRAP protocol

We performed surgery on wild type mice to insert MBH cannulae. After recovery of 8 – 12 days, mice were studied. Mice were fasted for 6 hours, before injection with either leucine or ACSF vehicle. After 45 minutes, mice were euthanized, brains were removed and MBH

dissected. MBH from 4 or 5 animals per treatment were pooled. This was to ensure sufficient RNA was collected in the immunoprecipitate, which leads to a 10 fold reduction in RNA compared to the total RNA (Knight et al., 2012). Pooling increases variability within each sample. In addition, if mishits with the surgery occurred, this dilutes the strength of the signal detected. In one cohort of animals, we confirmed via immunostaining that pS6 expression was increased 45 minutes after MBH leucine administration (data not shown). In the other animals, after the dissection, phosphorylated ribosome capture, by immunoprecipitation, was performed on half the tissue sample taken, with the other half used for the total RNA sample. RNA was extracted and prepared for RNA sequencing. After sequencing, FPKM values were calculated.

Initially, I assessed the technical success of the protocol. Neurotransmitters expressed in the arcuate or ventromedial nucleus were enriched relative to non-arcuate or VMN markers in the input samples, confirming the accuracy of the dissection (Figure 3-2A). The success of the immunoprecipitation was then confirmed, by assessing the fold enrichment of activity dependent genes, which should be enriched in i.p. samples (so will have high enrichment ratios). Although there was variability between the samples, the data demonstrate that fold enrichment of activity dependent genes is increased compared to the housekeeper control *Gapdh* Figure 3-2B.

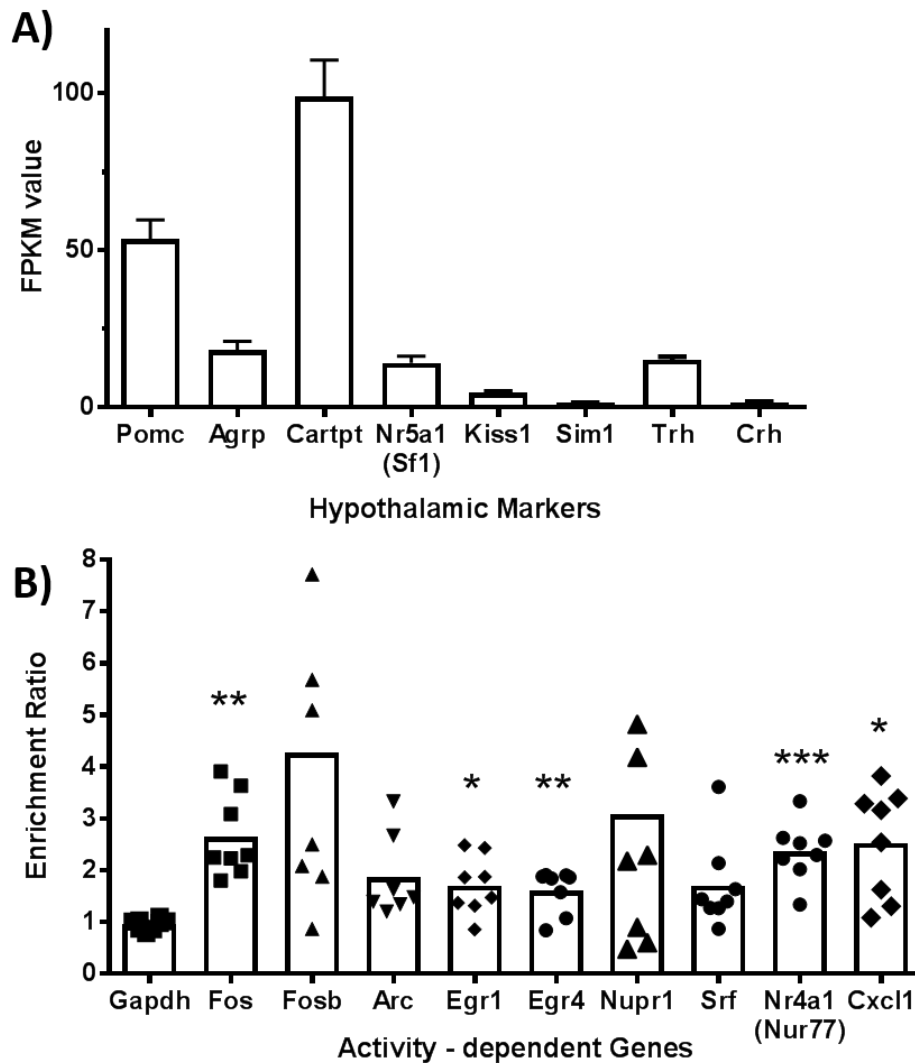


Figure 3-2: Confirmation of dissection success, and enrichment of activity dependent genes in *i.p.* samples.

A) Average FPKM values of hypothalamic markers from input samples and B) Enrichment of activity dependent genes in ACSF and leucine treated samples, each data point represents the enrichment ratio for the gene stated in one pooled sample, enrichment ratio: FPKM *i.p.*/FPKM input for the treatment group. Data in B) Analysed by One Way ANOVA, comparing all samples to GAPDH, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Genes: A) *Pomc* – pro – opio melanocortin, *Agrp* – agouti related peptide, *Cartp* – Cocaine- and amphetamine-regulated transcript peptide, *Nr5a1* – NR5A1 nuclear receptor subfamily 5 group A member 1 (encodes VMN neurotransmitter SF1), *Kiss1* – Kisspeptin, *Sim1* – Single-minded homolog 1 (PVH Marker), *Trh* – Thyrotropin-releasing hormone (PVH marker), *Crh* – Corticotropin-releasing hormone. B) *Gapdh* – Glyceraldehyde 3-phosphate dehydrogenase, *Fos* – Fos Proto-Oncogene, *Fosb* – Fos Proto-Oncogene b, *Arc* – activity-regulated cytoskeleton-associated protein, *Egr1* – Early Growth Response 1, *Egr4* – Early Growth Response 4, *Nupr1* – Nuclear Protein 1, Transcriptional Regulator, *Srf* – Serum response factor, *Nr4a1* – Nuclear Receptor Subfamily 4 Group A Member 1, *Cxcl1* – chemokine ligand 1.

3.4.2 Top candidate hits and pathways involved in leucine sensing

Candidate genes were identified by a screening process as listed in the methods (section 3.3.6). Initial filtering identified 478 genes; more stringent filtering identified 48 candidate genes. We subsequently studied this list to identify genes to test further. We looked for genes with enriched expression in leucine sensing brain regions (MBH and NTS) using the Allen Brain Atlas as a guide. We looked for genes known to be nutritionally regulated, using studies such as Henry et al., 2015 as a database to study. We also compared our list of genes to genes known to cause severe early onset obesity. These have been identified in patients in the GOOS (Genetics of Obesity Study) cohort, a group of over 5000 patients with rare early onset obesity, led by Professor Sadaf Farooqi (Hendricks et al., 2017).

A list of the candidates is presented in Table 3-1. The enrichment ratio calculation will always produce a positive value. Inhibited markers will have an enrichment ratio between 0 and 1. We took the reciprocal of these values, and made them negative for the purpose of this table so markers of leucine inhibited cells can be identified.

Calca, *Calcr* and *Ramp2* are components of the CGRP (Calcitonin gene-related peptide) signalling pathway. *Calca* encodes for peptides including CGRP, *Calcr* is the receptor for CGRP, a GPCR, and *Ramp2* is a protein that modifies *Calcr* signalling (McLatchie et al., 1998). This suggests CGRP Neuropeptide Release may play a role in leucine sensing. CGRP is a neurotransmitter that is expressed by a group of neurons in the parabrachial nucleus (PBN), that have been extensively studied by the Palmiter lab (Campos et al., 2016; Carter et al., 2013; Wu et al., 2009). A neurocircuit from the PBN to amygdala has been implicated in the suppression of appetite (Campos et al., 2016; Carter et al., 2013; Wu et al., 2009). We attempted to explore if leucine altered CGRP release, using a slice preparation model (Hussain et al., 2015). We took thick brain sections from mice, incubated them in culture media (used in Chapter 2), treated them with 500µM leucine, and collected the media to measure neurotransmitter release. However, when KCl was applied as a positive control at the end of the assay to depolarise all cells and cause neurotransmitter release, in this assay, we did not observe an increase in CGRP release (data not shown). This suggests the assay failed. We had previously demonstrated these assays did work for studying AgRP release, which was suppressed by leucine.

Of the remaining candidate genes identified, four stand out as of particular interest for further study. *Cacna1g*, a T type voltage gated calcium channel, is discussed further in the next section.

Cochlin (*Coch*) is a secreted protein that is involved in hearing and eyesight. Mutations in *Coch* are associated with deafness and glaucoma (Bae et al., 2014). Cochlin expression is enriched in the MBH (Allen Brain Atlas). In addition, cochlin is one of the top genes that is enriched in AgRP neurons after fasting, compared to fed AgRP neurons (Henry et al., 2015).

Tas1r1, encoding a GPCR implicated in amino acid sensing in the gustatory system (Nelson et al., 2002), has a negative enrichment score. *Tas1r1* can dimerize with another GPCR *Tas1r3* to sense amino acids, including leucine (Nelson et al., 2002). This suggests it may be involved in leucine sensing in leucine inhibited neurons.

Atp6v0a2 encodes a vacuolar ATPase, that has been implicated in amino acid sensing via the mTORC1 pathway (Hoxhaj et al., 2016). It has a positive ER, suggesting it may be necessary for leucine sensing in leucine activated neurons.

Table 3-1: Table listing some of the top candidate genes identified in Phospho TRAP study. First column, gene name, second column, enrichment ratio in phospho TRAP, third column, p – value from Cufflinks software.

Gene Abbreviation	Gene Name	Phospho TRAP ER (leu vs veh)	p-value
Cytoplasmic proteins			
<i>Dlec1</i>	Deleted In Lung And Esophageal Cancer Protein 1	-4.23	0.01
<i>Fmn13</i>	Formin-like protein 3	-2.65	0.01
<i>Atp6v0a2</i>	V-ATPase 116 kDa isoform a2	1.99	0.00
<i>Hyls1</i>	Hydrolethalus Syndrome Protein 1	2.00	0.00
<i>Stk36</i>	STK36 serine/threonine kinase 36	2.99	0.01
<i>Rnf125</i>	E3 Ubiquitin-Protein Ligase RNF125	4.24	0.00
<i>Dapk2</i>	Death Associated Protein Kinase 2	5.07	0.01
Secreted proteins			
<i>Fgf10</i>	Fibroblast growth factor 10	-2.23	0.00
<i>Calca</i>	Calcitonin Related Polypeptide Alpha	2.95	0.01
<i>Coch</i>	Cochlin	3.69	0.02
<i>Vash2</i>	Vasohibin 2	8.70	0.00
GPCR			
<i>Chrm1</i>	Muscarinic acetylcholine receptor M1	-3.12	0.02
<i>Tas1r1</i>	Taste receptor type 1 member 1	-1.96	0.00
<i>Calcr</i>	Calcitonin Receptor	3.22	0.01
Nucleus			
<i>Msh5</i>	MutS Homolog 5	-4.41	0.00
<i>Creb3L1</i>	cAMP Responsive Element Binding Protein 3 Like 1	2.64	0.01
<i>Cep110</i>	Centriolin	-2.20	0.01
Plasma Membrane			
<i>Ephb3</i>	Ephrin Type-B Receptor 3	-3.98	0.02
<i>Ramp2</i>	Receptor Activity Modifying Protein 2	-1.79	0.01
<i>Sfrp4</i>	Secreted frizzled-related protein 4	3.92	0.02
<i>Cacna1g</i>	Calcium channel, voltage-dependent, T type, alpha 1G subunit	4.52	0.01
<i>Ano6</i>	Small-conductance calcium-activated nonselective cation channel	2.27	0.01
<i>Tm9sf1</i>	Transmembrane 9 Superfamily Member 1	3.12	0.02
<i>Slc2a5</i>	GLUT5	4.47	0.01
<i>Ctns</i>	Lysosomal Cystine Transporter	3.69	0.01
<i>Mfsd10</i>	Major Facilitator Superfamily Domain Containing 10	3.79	0.00

Pathway analysis using Ingenuity Canonical Pathway Analysis software identified multiple pathways up or downregulated in leucine sensing cells (Table 3-2). The mTOR and p70S6K signalling pathways were upregulated. Although these pathways are known to be involved in

MBH leucine sensing (Blouet et al., 2008, 2009), it is likely these are enriched due to the technique enriching for cells with pS6, downstream of mTORC1 and p70S6K1 (Knight et al., 2012). The AMPK signalling pathway was downregulated by leucine, which may be a result of inhibition by the mTOR pathway. Erk signalling was also activated, a known signalling pathway in MBH leucine sensing (Blouet et al., 2009).

The analysis identified multiple pathways related to actin polymerization that were regulated in leucine sensing cells. This was a pathway I explored during my PhD. Leucine increased actin expression in neuron cultures, assessed with western blot, although subsequent experiments were not conclusive (data not shown). Additionally, leucine treatment increased the extension of actin processes, and radial structures around the cytoplasm, in neuron cultures (data not shown). However, further repeats were not performed due to time constraints. These studies are being continued by other lab members using neuronal cell lines as a more convenient model to study leucine regulation of this pathway.

Table 3-2: Pathway analysis using Ingenuity canonical pathway analysis.

The z – score indicates pathway enrichment (positive – increased pathway expression/upregulated, negative – decreased pathway expression/downregulated).

Ingenuity Canonical Pathways	z-score	Function
p70S6K Signaling	1.134	cell cycle, growth, proliferation
ERK/MAPK (Mitogen-activated protein kinase) Signaling	1.265	
mTOR Signaling	1.89	
PI3K (Phosphoinositide 3-kinase) Signaling	2.449	
AMPK Signaling	-0.302	cellular energy homeostasis
RhoGDI (Rho GDP-dissociation inhibitor) Signaling	-0.832	actin polymerization
RhoA (Ras homolog gene family, member A) Signaling	0.333	
Regulation of Actin-based Motility by Rho	0.378	
Signaling by Rho Family GTPases	1.155	
TNFR1 (Tumor necrosis factor receptor 1) Signaling	-1.342	apoptosis inflammation
Ceramide Signaling	-1.134	apoptosis, differentiation, proliferation
TGF (Transforming growth factor)-beta Signaling	0.447	
HIPPO (Salvador/Warts/Hippo (SWH) pathway) signaling	1.342	
Wnt/beta-catenin Signaling	-0.333	proliferation, differentiation
PDGF (Platelet-derived growth factor) Signaling	1	
FGF (fibroblast growth factor) Signaling	1.342	
Growth Hormone Signaling	2	
Integrin Signaling	0.9	cell-cell, cell-ECM interaction
Ephrin Receptor Signaling	0.447	axon guidance, cell-cell interaction
GDNF (Glial cell line-derived neurotrophic factor) signaling	2	neuronal growth/survival
NGF (Nerve growth factor) Signaling	2.449	
CDK5 (Cyclin-dependent kinase 5) Signaling	0.447	neuronal maturation and migration
Dendritic Cell Maturation	0.816	signal transduction, cytoskeleton, nerve guidance, membrane potential
Dopamine Signaling	2.236	
Purinergic Receptor Signaling	2.236	

3.4.3 T Type Calcium Channels play a role in leucine sensing, and are required for leucine sensing in POMC Neurons

One of the candidate genes was a calcium channel which the phospho TRAP data set shows is enriched in leucine activated neurons Table 3-1. *Cacna1g* encodes a T Type calcium channel. We sought to test if this channel was necessary for the rapid calcium response to leucine identified in chapter 2. T Type calcium channels generate distinct currents in neurons that can be identified using electrophysiology. A relatively new, specific, inhibitor was identified by its ability to block these currents (Choe et al., 2011; Dreyfus et al., 2010). This inhibitor was used in calcium imaging experiments to test if TTA – P2 reduced the percentage of leucine sensing cells (at a concentration recommended in the studies previously performed, 3 μ M). Some neurons still responded to leucine in the presence of TTA – P2 (Figure 3-3B and C) but the percentage was reduced, (Figure 3-3D). In POMC neurons, TTA – P2 abolished the response to leucine (Figure 3-3E). This data demonstrates that T Type calcium channels are necessary for leucine sensing in POMC neurons, and a group of non – POMC leucine sensing neurons.

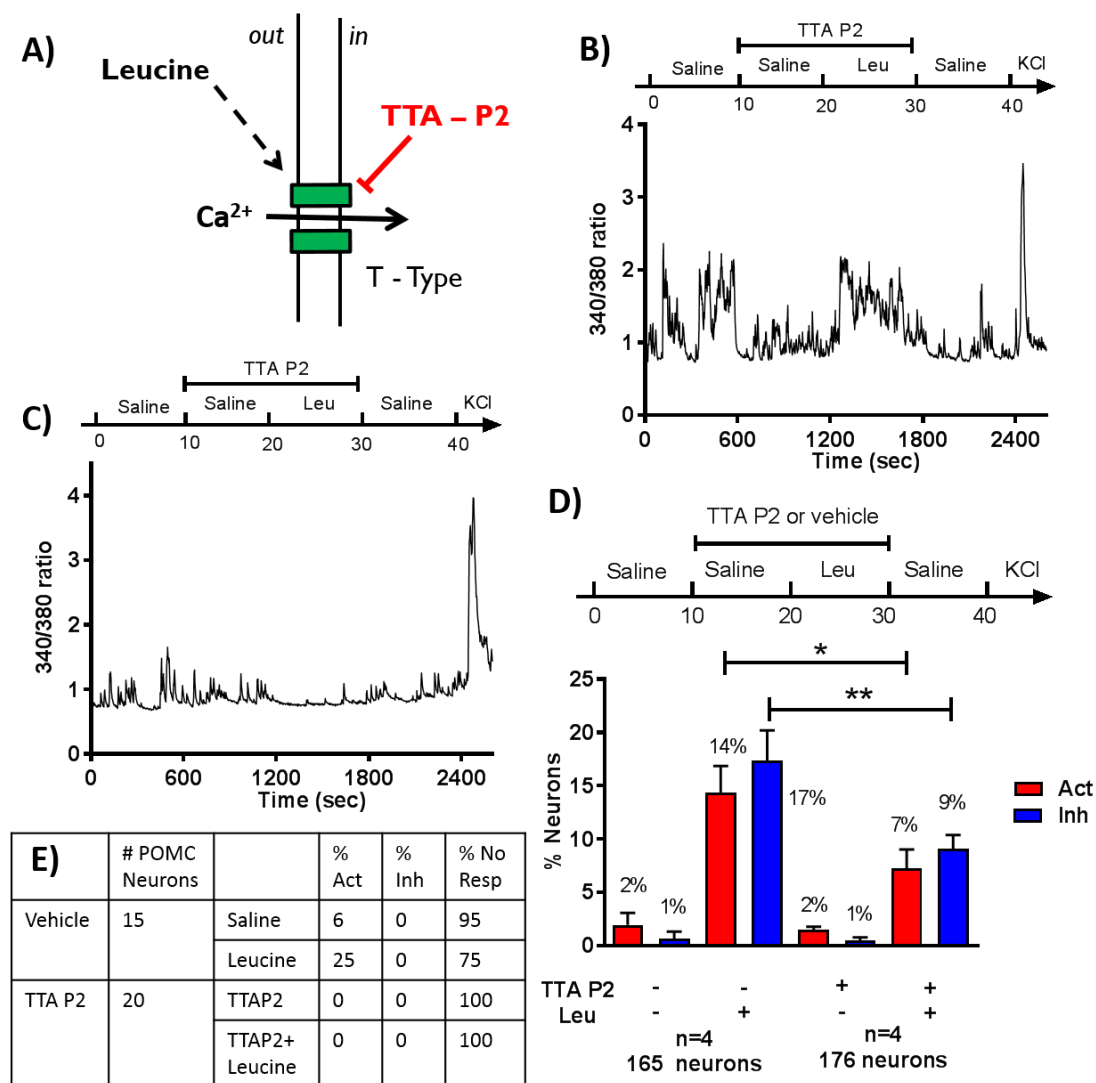


Figure 3-3: T Type calcium channel inhibition reduces number of leucine sensing cells.

A) Schematic demonstrating action of TTA – P2, dashed line indicates unknown link, B) Trace of neuron activated by leucine in presence of 3 μ M TTA – P2, C) Trace of neuron inhibited by leucine in presence of 3 μ M TTA – P2, D) Graph showing percentage of responsive neurons in different treatments, data analysed by Two Way ANOVA with Tukey's post hoc test, E) Summary table of responses of POMC neurons to TTA – P2, * $p < 0.05$, ** $p < 0.01$.

3.4.4 Single Cell RNA Sequencing of leucine sensing cells

Leucine is sensed by heterogeneous mechanisms. Leucine sensing that generates the rapid calcium response observed in chapter 2 may rely on different mechanisms to those that may be identified from the phospho TRAP study. Although T Type calcium channels are involved in the response to leucine, some neurons still respond to leucine in the presence of the T Type channel inhibitor TTA – P2 (Figure 3-3D). It is not clear how these T Type channels are activated or inhibited downstream of leucine sensing.

In an attempt to gain further understanding of the leucine sensing population, and to identify markers for these neurons, after calcium imaging neurons, the cells were collected into a pipette, lysed, and single cell RNA sequencing was performed (sequencing according to (Lam et al., 2017)).

3.4.5 Identification of cell types

Using an RNA sequencing transcriptome reference database of cells expressed in the cerebral cortex (Zhang et al., 2014), cells were assessed to identify if they were neuronal, or another brain cell type. Commonly used markers, such as NeuN and MAP2 for neurons, and GFAP for astrocytes, were also included in the analysis, although these were not all identified as top markers in the expression database (Zhang et al., 2014). Top marker genes, chosen for high FPKM values, and a high FPKM value relative to other groups, were used to classify cells, and plotted on a heatmap (Figure 3-4). Most cells expressed markers of neurons, with low expression of markers of immature neurons. The data are presented as heatmaps. Each square represents \log_2 of the FPKM value of the gene in an individual cell, blue indicates low or no expression, red indicates high expression.

The POMC and NPY GFP neurons, labelled POMC and NPY, serve as useful tools to identify markers that are reliably expressed in neurons. NeuN (*Rbfox3*) was not expressed in all POMC or NPY neurons, and MAP2 expression was variable. Genes identified from the expression database (Zhang et al., 2014) as neuronal markers, *Tmem130*, *Ptprn*, *Resp18*, *Pcp4* and *Tmem691*, were expressed highly in POMC and NPY cells – with the exception of POMC Con 0 which did not express *Ptprn* and *Tmem130*.

Activated cells 2 and 6 expressed some markers of newly formed oligodendrocytes. Inhibited cell 2 expressed markers of newly formed oligodendrocytes, myelinating oligodendrocytes and astrocytes. Lower expression of most of the neuronal genes in Act 6 and Inh 2 suggests they may be non-neuronal. Act 2 appears to express most of the neuronal markers, demonstrating the importance of using multiple markers to classify cells. Control cells 2 and 3, although expressing some neuronal markers (*Resp18*, *Pcp4* and *Tmem130*), had lower expression of others such as *Ptprn* and *Tmem691*.

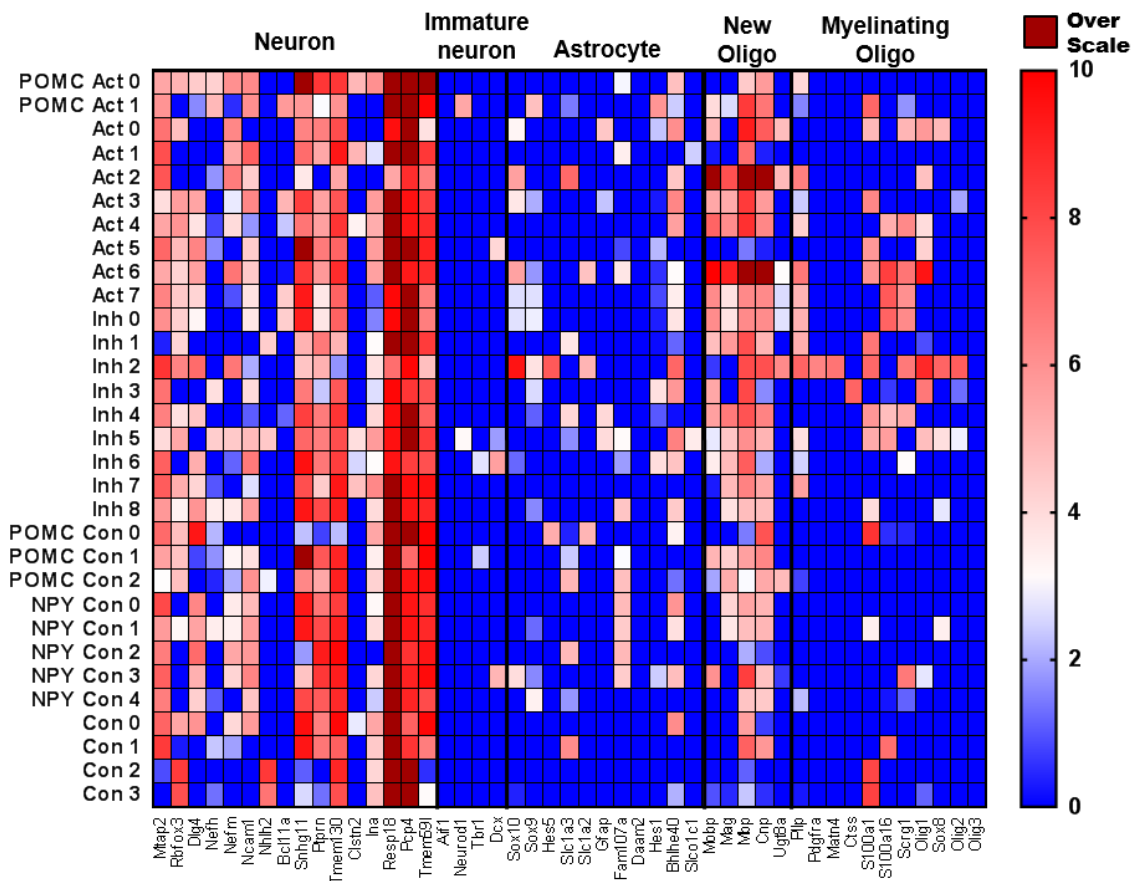


Figure 3-4: Heatmap to identify cell type based on markers in Zhang et al (2014).

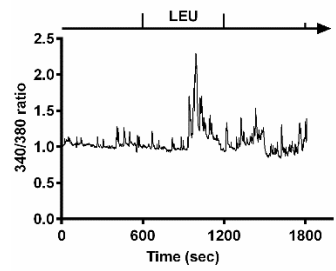
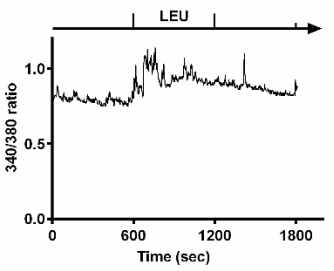
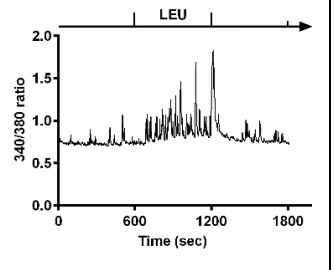
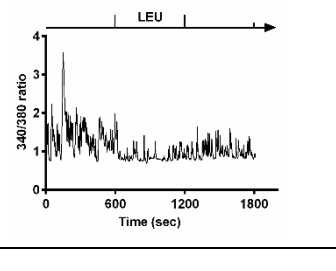
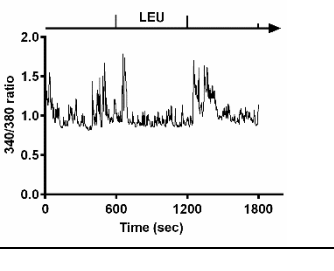
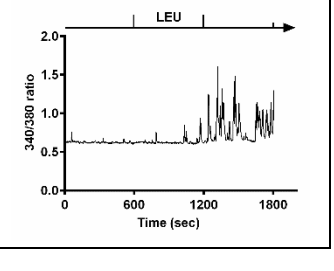
Heatmap for markers of Neurons, immature neurons, astrocytes, new oligodendrocytes and myelinating oligodendrocytes, on a log2 scale. White represents FPKM = 5. Brown values are readings above the range of the scale. Mtap2 - Microtubule-associated protein 2, Rbfox3 - Neuronal Nuclei (NeuN), Dlg4 - postsynaptic density protein 95, Nefh and m - Neurofilament Heavy and Medium, Ncam1 - Neural Cell Adhesion Molecule 1, Nhlh2 - Nescient Helix-Loop-Helix 2, Bcl11a - B-cell lymphoma/leukemia 11A, Snhg11 - Small Nucleolar RNA Host Gene 11, Ptpn - Protein Tyrosine Phosphatase, Receptor Type N, Tmem130 and 59l - Transmembrane Protein 130 and 59 like, Clstn2 - Calsyntenin 2, Ina - Internexin Neuronal Intermediate Filament Protein Alpha, Resp18 - Regulated Endocrine Specific Protein 18, Pcp4 - Purkinje Cell Protein 4, Aif1 - Allograft Inflammatory Factor 1, Neurod1 - Neuronal Differentiation 1, Tbr1 - T-Box, Brain 1, Dcx - Doublecortin, Sox 8 - 10 - SRY-Box 8 - 10, Hes5 and 1 - Hes Family BHLH Transcription Factor 5 and 1, Slc1a3 and 2 - Excitatory amino acid transporter 1 and 2, Gfap - Glial Fibrillary Acidic Protein, Fam107a - Family With Sequence Similarity 107, Daam2 - Dishevelled Associated Activator Of Morphogenesis 2, Bhlhe40 - basic helix-loop-helix family member e40, Slco1c1 - Solute carrier organic anion transporter family member 1C1, Mbbp - Splicing Factor 1, Mag - Myelin Associated Glycoprotein, Mbp - myelin basic protein, Cnp - 2',3'-cyclic nucleotide 3' phosphodiesterase, Ugt8a - UDP galactosyltransferase 8A, Plip - Plasmalipin, Pdgfra - Platelet Derived Growth Factor Receptor, Matn4 - Matrilin 4, Ctss - Cathepsin S, S100a1 and 16 - S100 Calcium Binding Protein A1 and 16, Scrg1 - Stimulator Of Chondrogenesis 1, Olig 1 - 3 - Oligodendrocyte Transcription Factor 1 - 3

3.4.6 Neurochemical Identity of Single Cells

The response profiles of the captured neurons varied. Consequently, the cells were sub categorised, as outlined in Table 3-3. All heatmaps are presented with neurons in these subgroups.

Table 3-3: Summary of sub groups of responses of activated and inhibited neurons.

Table shows traces of neurons in each category, a description of the response, and the label (in bold) which is used on the heatmaps.

Category	Sub Group		
Activated	Peak Large Peak 	AUC Increase in Area under the curve 	Small Peaks Series of small peaks 
	Start Active Saline 1, leucine inhibits, no/small return to activity in saline 2 	Both, rebound Active Saline 1, leucine inhibits, active/large rebound in saline 2 	Rebound No response in Saline 1, no response in leucine, active/large rebound in saline 2 

The neurochemical identity of the neurons captured was confirmed, by assessing expression of known neurotransmitters (from the MBH and other hypothalamic nuclei) in the captured neurons (Figure 3-5). Unexpectedly, two of the captured POMC neurons, POMC Act 0 and POMC Con 0 in Figure 3-4, were not *Pomc* positive. POMC Act 0 was actually an *Agrp/Npy* neuron, and has been re labelled NPY Act 0 in Figure 3-5. POMC Con 0 is not clearly identifiable as one type of neuron. The other POMC and NPY GFP neurons were *Pomc* and *Agrp/Npy* positive, respectively. One inhibited neuron, captured from a POMC – GFP mouse, Inh 8, was an AgRP/NPY neuron, and has been relabelled NPY Inh 0. Many neurons

expressed *Npy*, *Avp* and *Sst*, suggesting neurons from anterior parts of the hypothalamus may have been captured, and may be leucine sensing.

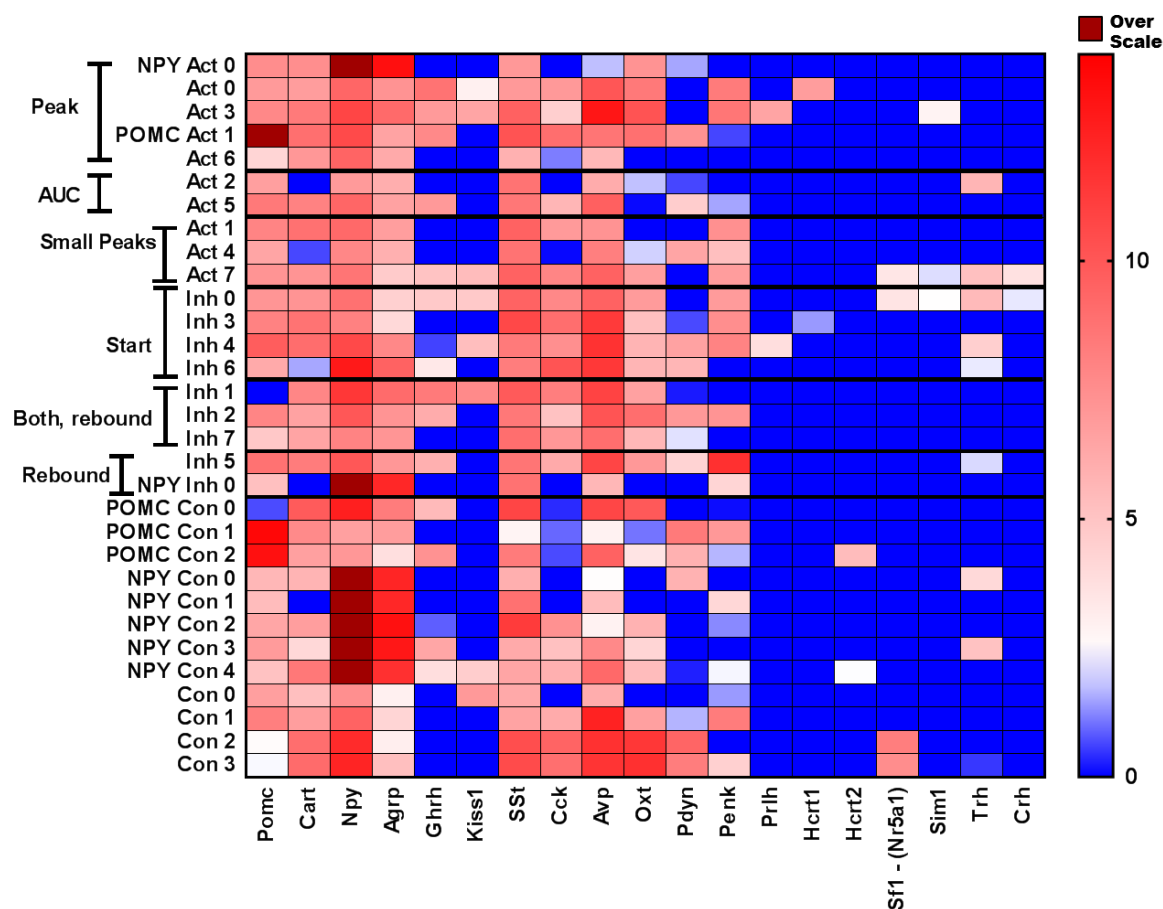


Figure 3-5: Heatmap for neurotransmitters in the hypothalamus

White represents FPKM = 5. Brown values are readings above the range of the scale. Plotted on a log2 scale. *Pomc* – pro – opio melanocortin, *Cartp* - Cocaine- and amphetamine-regulated transcript peptide, *Npy* – neuropeptide Y, *Agrp* – agouti related peptide, *Ghrh* - Growth hormone–releasing hormone, *Kiss1* – Kisspeptin, *Sst* – somatostatin, *Cck* – Cholecystokinin, *Avp* – vasopressin, *Oxt* – oxytocin, *Pdyn* – prodynorphin, *Penk* – Proenkephalin, *Prlh* - Prolactin Releasing Hormone, *Hcrt* – hypocretin, *Nr5a1* - NR5A1 nuclear receptor subfamily 5 group A member 1 (encodes VMN neurotransmitter SF1), *Sim1* - Single-minded homolog 1 (PVH Marker), *Trh* - Thyrotropin-releasing hormone (PVH marker), *Crh* - Corticotropin-releasing hormone.

Some hypothalamic neurons are cholinergic or dopaminergic (Meister et al., 2006; Qiu et al., 2006). Markers for these neurons were assessed (Figure 3-6). No neurons expressed *Chat*, the enzyme necessary for acetylcholine synthesis. However, 3 neurons had very high

expression levels of *Th*, the enzyme necessary for dopamine synthesis, and 4 others also expressed *Th*. All of the *Th* positive neurons were either activated or inhibited by leucine, not control neurons. Other candidate markers of dopaminergic neurons were assessed (Cossette et al., 2005; Hartfield et al., 2014; Reyes et al., 2012; White and Thomas, 2012). Dopa decarboxylase (*Ddc*) was expressed in 6/7 *Th* positive neurons. The other markers gave less clear results, suggesting either these neurons are not dopaminergic, or the markers chosen are not reliable markers for dopaminergic neurons, or the genes have been downregulated. This data suggests a significant proportion of leucine sensing neurons are dopaminergic.

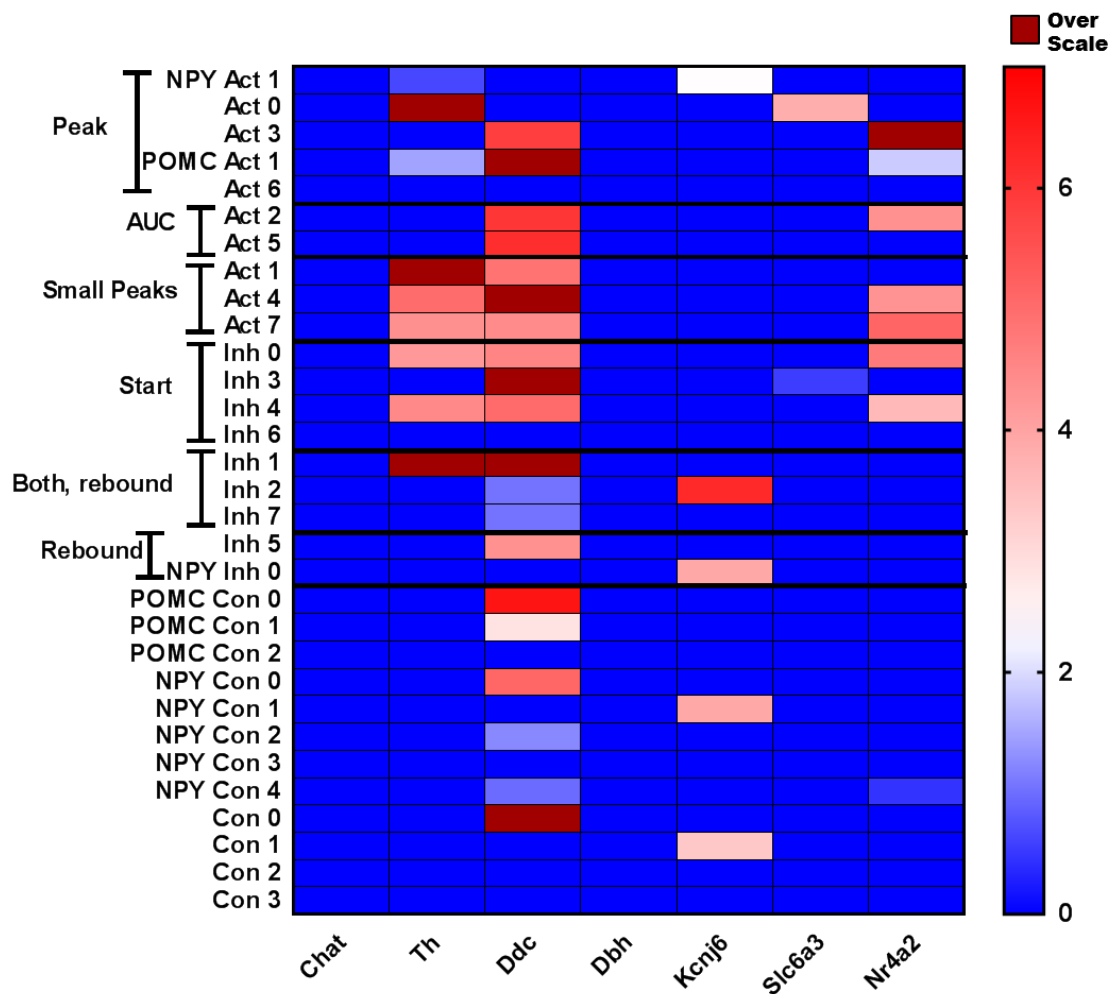


Figure 3-6: Heatmap for markers of cholinergic (*Chat*) and dopaminergic neurons.

White represents $FPKM = 5$. Brown values are readings above the range of the scale. Plotted on a \log_2 scale. *Chat* - choline *O*-acetyltransferase, *Th* – tyrosine hydroxylase, *Ddc* - dopa decarboxylase, *Dbh* - dopamine beta-hydroxylase, *Kcnj6* - Potassium Voltage-Gated Channel Subfamily J Member 6, *Slc6a3* - Solute Carrier Family 6 Member 3 (dopamine transporter), *Nr4a2* - Nuclear receptor related 1 protein.

Campbell et al., 2017 identified multiple, previously undescribed, populations of neurons in the arcuate nucleus of the hypothalamus, in addition to dividing known populations into subpopulations. Each population was found to have one or two principal markers (Figure 3 in (Campbell et al., 2017)). The expression of the top genes, identified as markers for each population, were plotted (Figure 3-7).

The POMC neuron markers *Ttr*, *Anxa2* and *Glpr1* can be used to classify POMC Act 1 and POMC Con 1, but not POMC Con 2 (no expression of any of the markers). The 7 AgRP/NPY

neurons can be subdivided based on *Sst* and *Gm8773* expression. *Sst* is expressed by all cells, but the expression varies. AgRP/NPY cells that express *Gm8773* express lower levels of *Sst* than cells that do not express *Gm8773*. *Th* cells can be subdivided based on *Sst*, *Nfib* and *Slc6a3* expression. Only 1 *Th* neuron expresses *Slc6a3*, and none express *Nfib*. The *Slc6a3* *Th* positive neuron expressed less *Sst* than the other 6 *Th* neurons. Some VMN markers were identified in Campbell et al., 2017, including *Rgs16*, *Bdnf* and *Nr5a1* (Sf1), which are expressed by some leucine sensing and control cells. It is more challenging to categorise other neurons as many express multiple markers. Using the bioinformatics tools developed by Campbell et al., 2017 may aid in this categorisation. It appears though that leucine sensing neurons are a highly heterogeneous population.

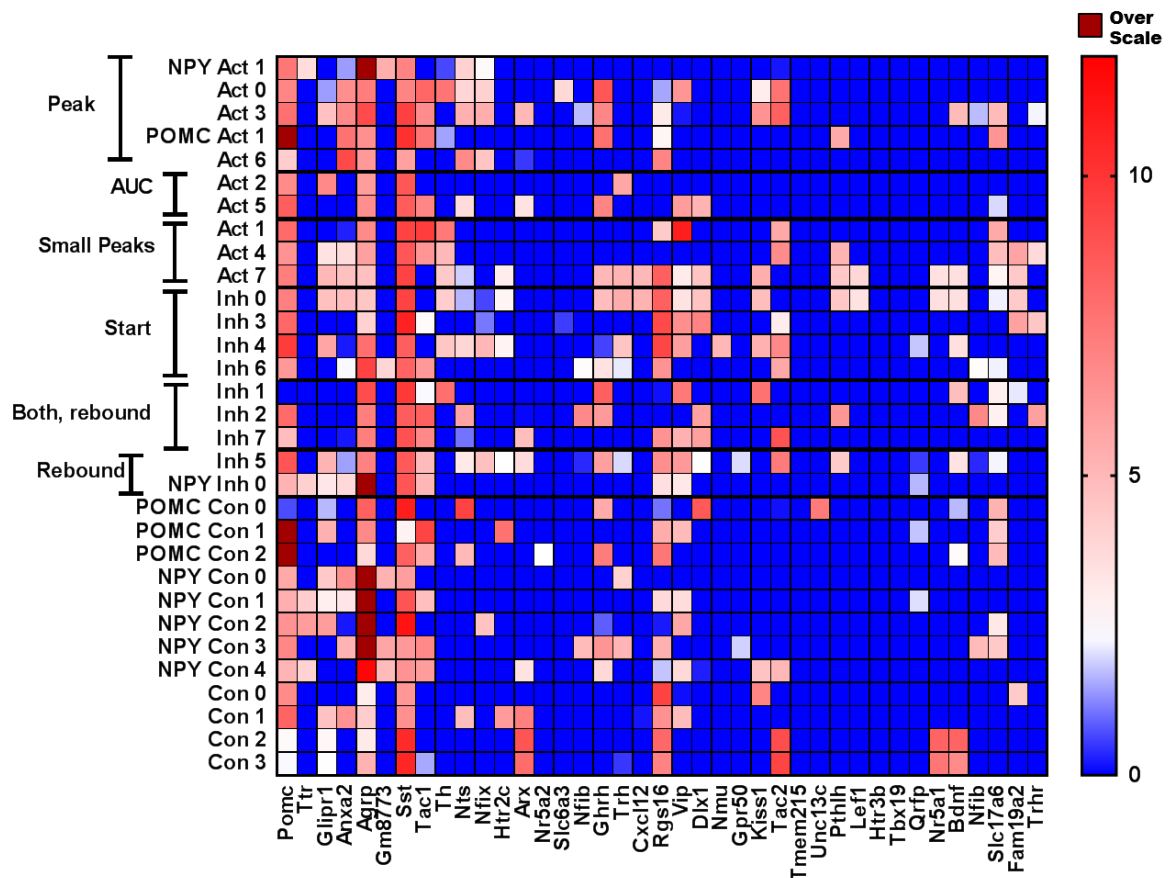


Figure 3-7: Heatmap for markers of MBH neurons as identified by Campbell et al 2017.

White represents FPKM = 5. Brown values are readings above the range of the scale. Plotted on a log₂ scale. Pomc – pro – opio melanocortin, Ttr – Transthyretin, Glipr1 - Glioma pathogenesis-related protein 1, Anxa2 - Annexin A2, Agrp – agouti related peptide, Gm8773 – predicted gene 8773, Sst – somatostatin, Tac1 - Tachykinin Precursor 1, Th – tyrosine hydroxylase, Nts – Neurotensin, Nfix - Nuclear Factor IX, Htr2c and 3b – serotonin 2C and 3B receptor, Arx - Aristaless-Related Homeobox, Nr5a1 and 2 - nuclear receptor subfamily 5 group A member 1 and 2, Slc6a3 - dopamine transporter, Nfib - Nuclear Factor 1 B-Type, Ghrrh - Growth Hormone Releasing Hormone, Trh - thyrotropin releasing hormone, Cxcl12 - Stromal cell-derived factor 1 precursor, Rgs16 - Regulator Of G Protein Signaling 16, Vip - Vasoactive Intestinal Peptide, Dlx1 - Distal-Less Homeobox 1, Nmu - Neuromedin U, Gpr50 - G Protein-Coupled Receptor 50, Kiss1 – kisspeptin, Tac2 - tachykinin 2, Tmem215 - Transmembrane Protein 215, Unc13c - Munc13-3, Pthlh - Parathyroid Hormone Like Hormone, Lef1 - Lymphoid Enhancer Binding Factor 1, Tbx19 - T-Box 19, Qrfp - pyroglutamylated RFamide peptide, Bdnf – brain derived neurotrophic factor, Slc17a6 – Vesicular Glutamate Transporter 2, Fam19a2 - Chemokine-Like Protein TFA-2, Trhr - thyrotropin-releasing hormone receptor

3.4.7 Expression of genes related to nutrient sensing in the MBH

As discussed in chapter 1, studies have attempted to sub divide POMC neurons based on expression of markers such as the leptin, insulin, and serotonin 2C receptors (Lam et al., 2017; Sohn and Williams, 2012; Sohn et al., 2011). For instance, leptin and the serotonin 2C receptor were demonstrated in electrophysiology studies to be expressed in distinct POMC neurons (Sohn et al., 2011). In contrast, in the cells here, one POMC neuron expressed both receptors (POMC Con 1) (Figure 3-8).

Other GPCR's are known to play important roles in the control of energy homeostasis, such as MC4R and NPY receptors (Yeo and Heisler, 2012). Their expression was evaluated and plotted. There was no noticeable enrichment of any of these known GPCR's in leucine sensing cells (data not shown).

A novel subpopulation of hypothalamic neurons was recently described, that when activated optogenetically, rapidly caused satiety (Fenselau et al., 2017). These neurons were demonstrated to express the oxytocin receptor. However, in this data set, only 1 leucine sensing neuron expressed this receptor. Leucine may not regulate this subpopulation to drive satiety.

A sodium channel, Nav1.7, encoded by the gene *Scn9a*, was recently described to be important in the ability of hypothalamic neurons to sum synaptic inputs over a long time duration (Branco et al., 2016). Sustained opening of this channel prevented membrane potential decay, allowing inputs to be summed over time. This channel was expressed in most of the AgRP/NPY neurons, but there was no specific enrichment in leucine sensing cells.

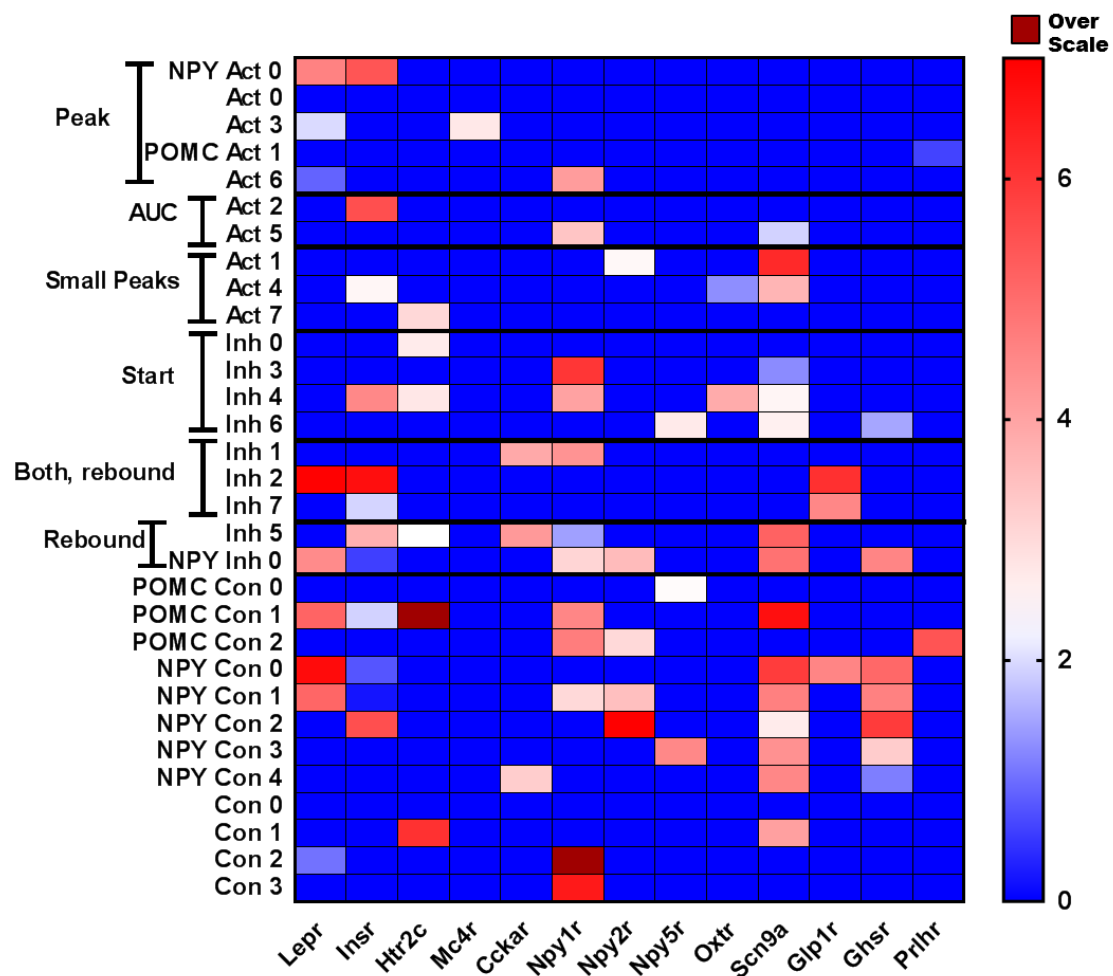


Figure 3-8: Heatmap for commonly studied genes of interest in hypothalamic nutrient and hormone sensing

White represents FPKM = 5. Brown values are readings above the range of the scale. Plotted on a log2 scale. *Lepr* – leptin receptor, *Insr* – insulin receptor, *Htr2c* – serotonin 2C receptor, *Mc4r* – melanocortin 4 receptor, *Cckar* – CCK receptor, *Npy1,2* and *5r* – Npy receptor types 1, 2 and 5, *Oxtr* – oxytocin receptor, *Scn9a* – voltage gated sodium channel 1.9, *Glp1r* – GLP – 1 receptor, *Ghslr* – ghrelin receptor, *Prlhr* - Prolactin-releasing peptide receptor

3.4.8 *Cacna1g* is not expressed in all leucine sensing single neurons

Given that *Cacna1g* was shown in the phospho TRAP study to be enriched in leucine sensing cells, I examined if the single leucine sensing cells expressed this channel (Figure 3-9). While *Cacna1g* is expressed in some leucine activated and inhibited cells, it is also expressed in control cells. This suggests *Cacna1g* may not be a specific marker for leucine sensing neurons, but is used by some to sense leucine. This is consistent with the pharmacological data, where the percentage of leucine sensing cells was reduced but not abolished.

Surprisingly, in the two GFP positive leucine sensing neurons (NPY Act 1 and POMC Act 0), *Cacna1g* was not expressed.

TTA – P2 will inhibit other T type calcium channels (*Cacna1h* and *i*) but, these channels are lowly expressed in the captured single cells. No other calcium channel shows specific enrichment in leucine sensing cells.

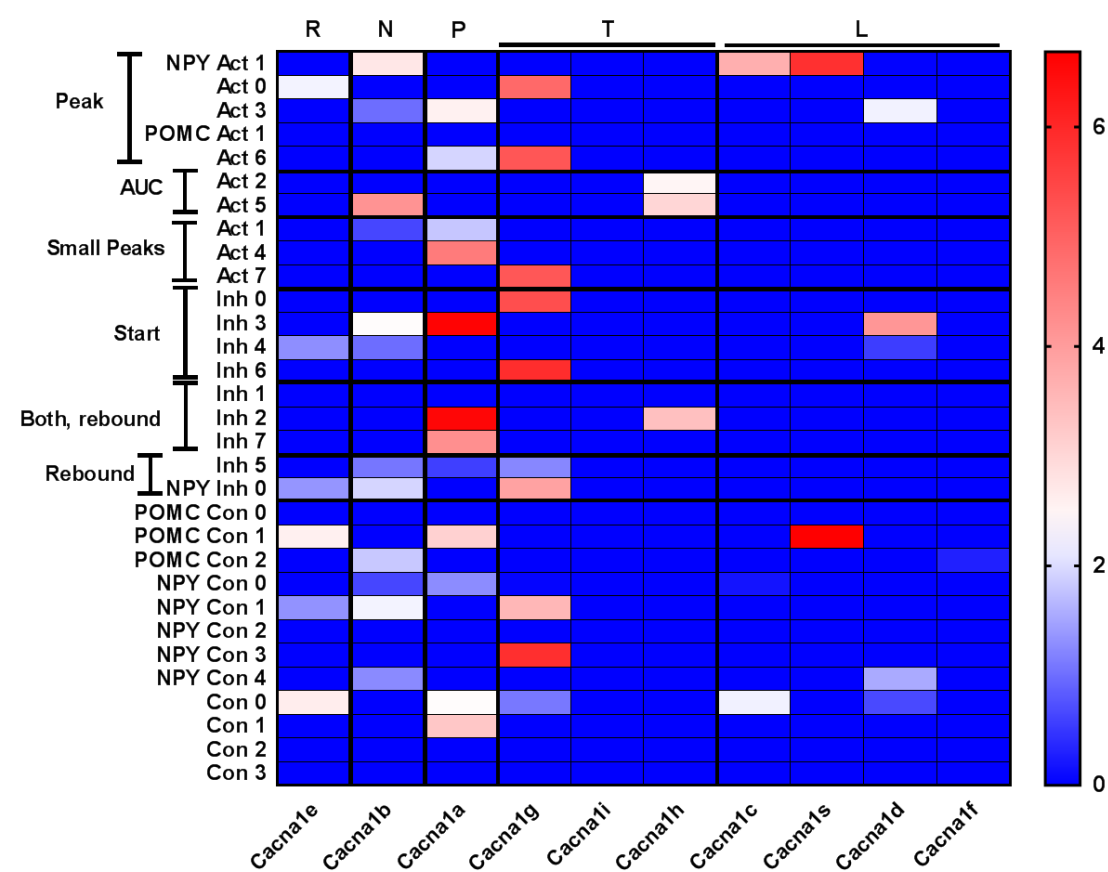


Figure 3-9: Heatmap for voltage gated calcium channels
Data plotted on a log2 scale. White represents FPKM = 5. Letters above stand for different classes of voltage gated calcium channels.

3.4.9 Low expression of ryanodine and IP₃ receptors in leucine sensing neurons

Data in chapter 2 suggested leucine sensing recruited intracellular calcium stores to initiate a calcium response, but studying the channels that regulate release of calcium from ER stores yielded unclear results. The expression of these channels in the captured neurons was assessed (Figure 3-10). 6 of the leucine sensing cells assessed expressed one of the subunits of one of the receptors (expression defined as FPKM above 5).

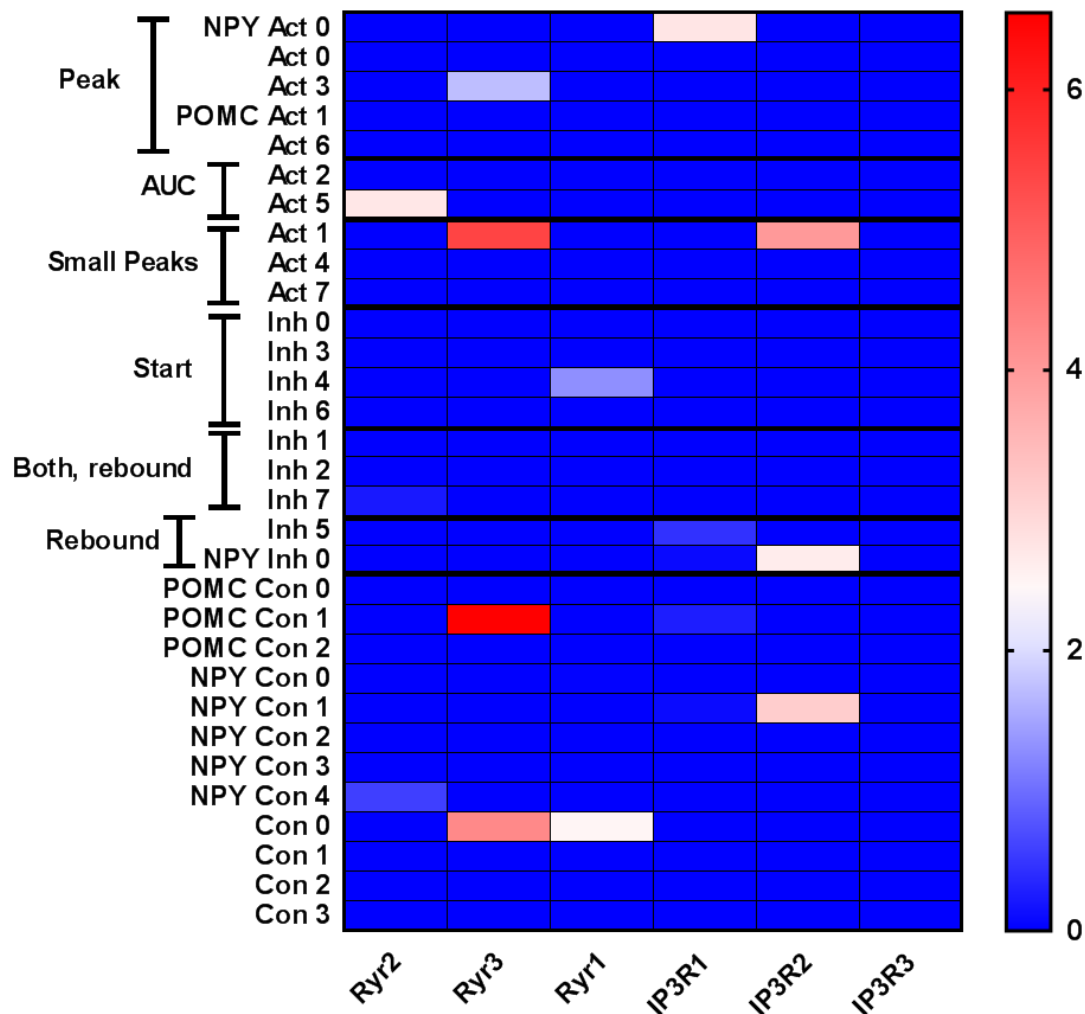


Figure 3-10: Heatmap for ryanodine and IP₃ receptors.

Data plotted on a log₂ scale. White represents FPKM = 5.

3.4.10 Screening Phospho TRAP hits in single cell sequencing data set

I examined if any of the hits from the phospho TRAP study were enriched in the leucine sensing cells that were captured (Figure 3-11). Besides *Ramp2*, most of the phospho TRAP hits with a negative enrichment ratio (suggesting that they are markers of leucine inhibited neurons) were not expressed in the captured neurons. There was no expression of *Tas1r1*, one of the negatively enriched phospho TRAP genes implicated in amino acid sensing in the gustatory system, or of *Tas1r3*, the GPCR it dimerizes with to sense amino acids in the gustatory.

For the markers that had a positive enrichment ratio in the phospho TRAP study, more of the hits were expressed in more of the captured cells. However, there was no marker that was

The facilitative glucose transporters SGLT1 and 2, implicated in glucose sensing in rat hypothalamic neurons (O'Malley et al., 2006), were expressed in 4 leucine sensing cells.

Glucose transporters GLUT1 – 6 have been implicated in glucose sensing in the brain and pancreas (Bady et al., 2006; Marty et al., 2006). GLUT3 was expressed in the majority of cells. There was no enrichment of any of the genes in leucine sensing cells.

The expression of the genes coding for the K_{ATP} channel was low, with only 3 leucine sensing cells expressing one of the genes necessary for a functional channel. This is in accordance with calcium imaging data showing few leucine sensing cells expressed K_{ATP} channels.

Expression of 6 genes involved in nitric oxide synthesis and sensing were assessed (Leshan et al., 2012; Murphy et al., 2009). Expression of the guanylyl cyclase *Gucy1b3* was broadest, but there was no noticeable enrichment of any of the genes in leucine sensing cells.

Taken together, this data suggests glucose sensing neurons overlap with some leucine sensing cells.

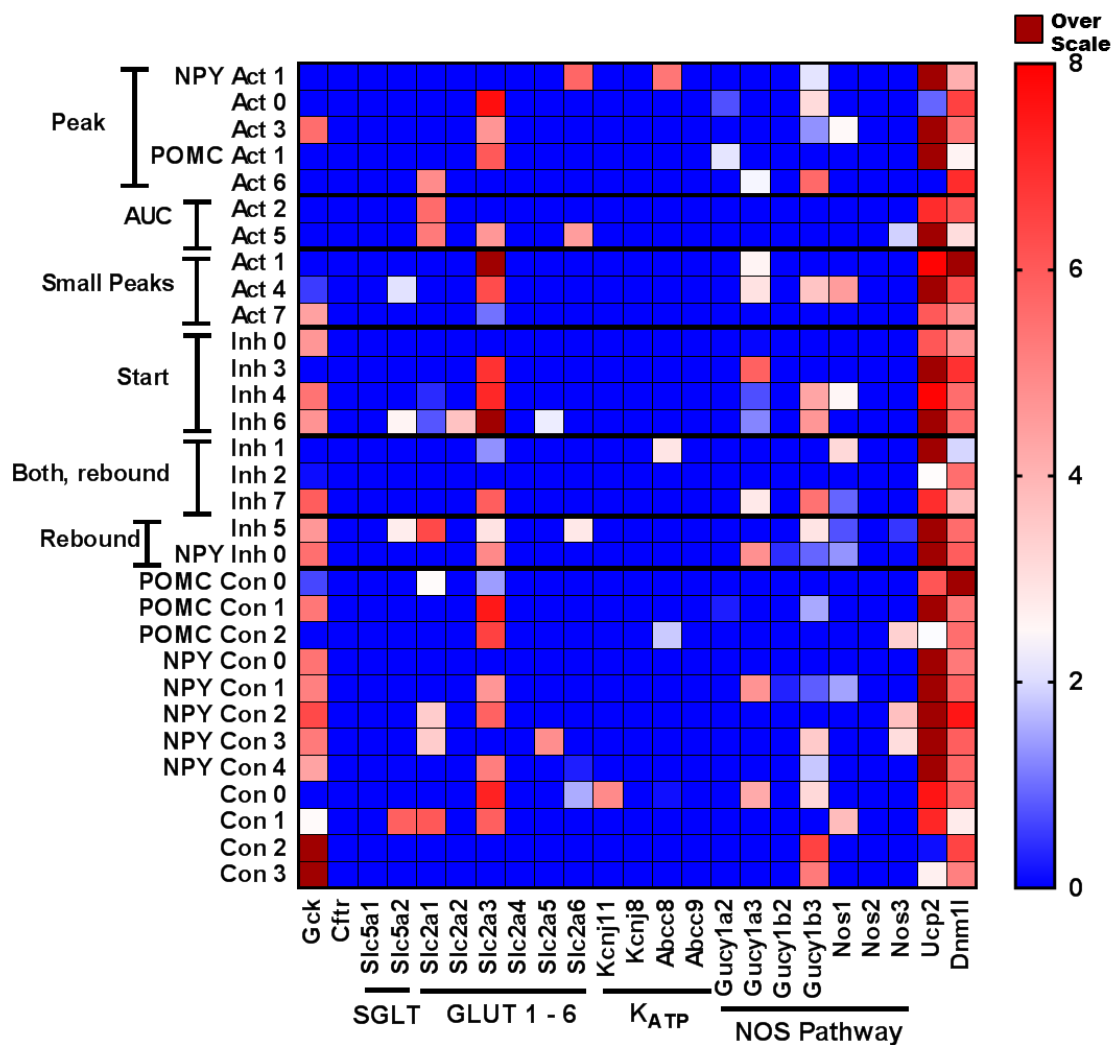


Figure 3-12: Heatmap for markers of glucose sensing neurons

Data plotted on a log2 scale. White represents FPKM = 5. Brown values are readings above the range of the scale. *Gck* – glucokinase, *Cftr* - Cystic fibrosis transmembrane conductance regulator, *SGLT* – sodium coupled glucose transporters, *GLUT* – glucose transporters, *Kcnj* - Potassium voltage-gated channel subfamily J, *Abcc* - ATP binding cassette subfamily C, *Gucy* - Guanylate cyclases, *Nos* – nitric oxide synthase, *Ucp2* – uncoupling protein 2, *Dnm1l* - Dynamin-1-like protein

3.4.12 Do leucine and fatty acid sensing cells overlap?

The expression of genes involved in hypothalamic fatty acid sensing was assessed in the leucine sensing neurons (Figure 3-13). The list of genes is taken from Le Foll et al., 2009 who studied the overlap of glucose and fatty acid sensing neurons, and performed single cell RT – PCR on candidate hits. This is not an exclusive list of all genes implicated in fatty acid sensing, and it is not clear if all of these genes in the table are involved in neuronal fatty acid sensing.

Cd36 and *Cpt1a*, previously identified as critical regulators of neuronal fatty acid sensing, were not expressed in the single cells sequenced (Figure 3-13) (Le Foll et al., 2009; Pocai et al., 2006). Of all genes assessed, *Slc27a1* and *Fasn* were expressed in more leucine sensing cells than control cells (Figure 3-13). *Fasn* has been implicated in hypothalamic fatty acid sensing (Cha et al., 2005; Chakravarthy et al., 2007; Landree et al., 2004).

The concept of metabolic sensing neurons, that could possibly respond to all three nutrients, has been proposed by the Levin lab (Levin, 2002, 2006; Levin et al., 2011). As *Cd36*, a key fatty acid sensing gene is not expressed in the single cells, *Fasn* and *Gck* expression was compared to see if the MBH single cells co – expressed these genes. 8 leucine sensing neurons expressed *Fasn*, and 8 leucine sensing neurons expressed *Gck*, but only 2 neurons expressed both.

Ucp2, implicated in both glucose and fatty acid sensing (Le Foll et al., 2009; Toda et al., 2016), is expressed in most single cells, but is not enriched in leucine sensing cells. K_{ATP} channels, implicated in MBH glucose, leucine, and oleic acid sensing (Le Foll et al., 2009; Kang et al., 2004; Miki et al., 2001; Su et al., 2012), as discussed earlier (section 3.4.11), were lowly expressed in the single cells (Figure 3-12).

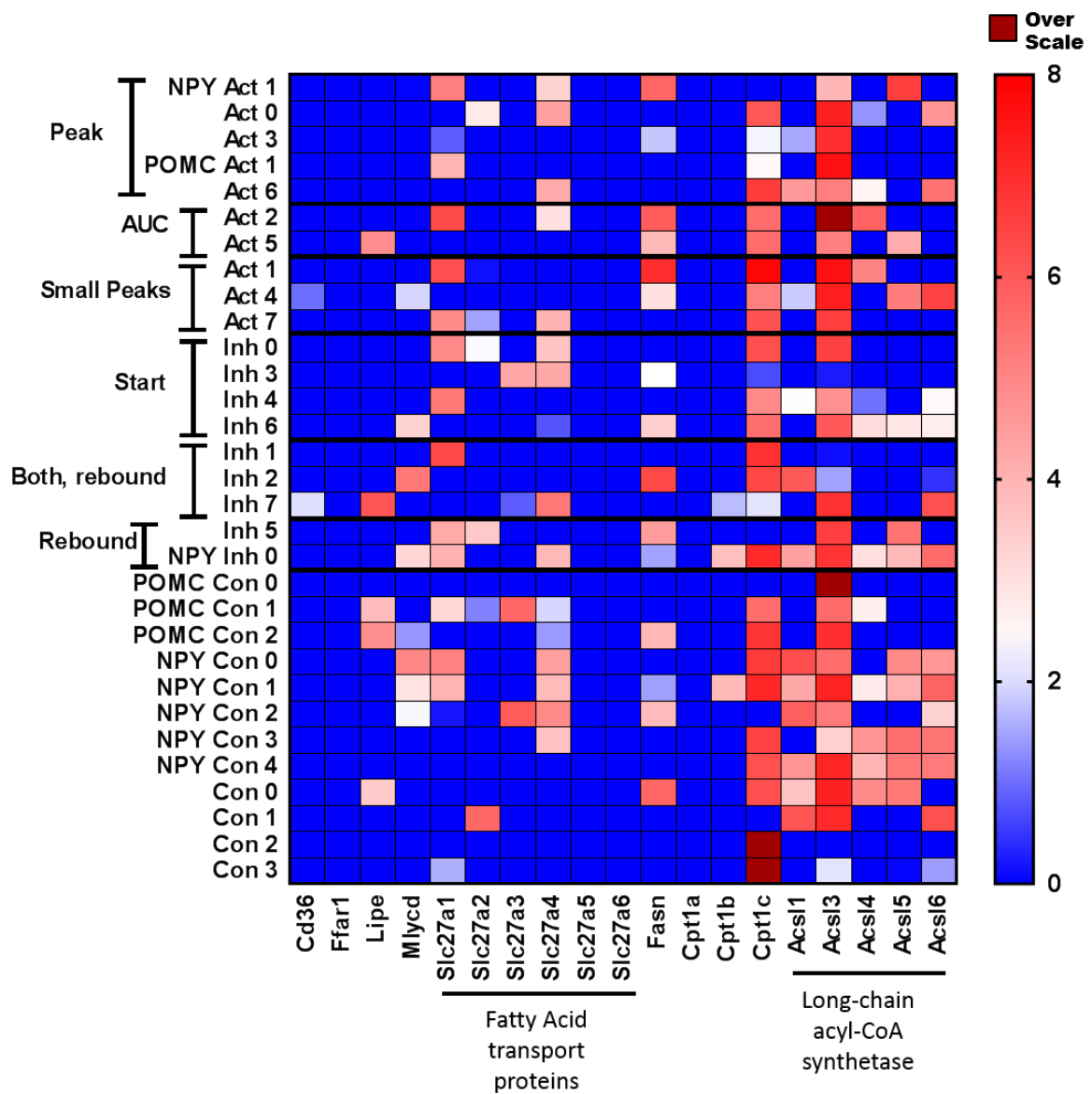


Figure 3-13: Heatmap for candidate markers of fatty acid sensing neurons

Data plotted on a log2 scale. White represents FPKM = 5. Brown values are readings above the range of the scale. Cd36 - fatty acid translocase, Ffar1 - Free fatty acid receptor 1, Lipe - lipase E (hormone sensitive type), Mlycd - Malonyl-CoA Decarboxylase, Fasn - Fatty Acid Synthase, Cpt1 - Carnitine palmitoyltransferase I

3.5 Discussion

3.5.1 Technical success of phospho TRAP

Several quality control checks were performed to confirm the phospho TRAP experiment was successful. Firstly, the FPKM values in the input samples of known MBH markers was assessed, to confirm dissection accuracy (Figure 3-2A).

Secondly, the enrichment ratio of activity dependent genes was confirmed, to show that the immunoprecipitation enriched for ribosomes found in activated neurons. For this, the enrichment ratios of genes known to be expressed in activated neurons was assessed. With the exception of *Fos*, the enrichment ratios for the other activity dependent genes were lower in our samples compared to those for a salt challenge presented in Knight et al., 2012. This could be because leucine is a milder stimulus compared to the salt challenge used by Knight et al., 2012. It could be an artefact of the pooling of brains. It could also suggest that our immunoprecipitation may not have been as successful at pulling down activated ribosomes. Another possibility is that the timepoint at which we collected the brains was not the optimal timepoint for pS6 activation. For cFOS immunostaining, brains are collected 80 – 100 minutes after stimulus/treatment, as it takes some time for cFOS protein to be produced and expressed in response to neuronal activation. The timecourse for pS6 as a marker for neuronal activation is less well defined, and the authors of Knight et al., 2012 advise performing experiments to assess the timecourse of pS6 expression after leucine administration. However, this is a costly and time consuming study that we did not perform, but could have been done in order to collect tissue at the point of maximum pS6 expression.

3.5.2 A role for T Type calcium channels in leucine sensing

One novel marker identified in the leucine sensing neurons from the phospho TRAP study was a T Type calcium channel gene, *Cacna1g*. Using the adult neuron culture model, the percentage of activated and inhibited leucine sensing cells was reduced when these channels were inhibited pharmacologically. Leucine sensing by POMC cells was abolished.

Given the calcium imaging results, a pilot *in vivo* study was performed by Clemence using TTA - P2, the inhibitor of T Type calcium channels. TTA – P2 was injected into the MBH of mice to see if it blocked the feeding effect of leucine. TTA – P2 blocked leucine's anorectic effect, but had no effect on feeding alone. These promising results require further confirmation, but suggest that this channel is an important component in a subpopulation of leucine sensing neurons that control feeding behaviour.

T type calcium channels are activated at lower voltages, and inactivate more slowly than other voltage gated calcium channels (Perez-Reyes, 2003). They have been demonstrated to play a role in burst firing of neurons, that facilitates neurotransmitter release (Perez-Reyes, 2003; Qiu et al., 2006; Zhang and van den Pol, 2015). Multiple signalling pathways have been

implicated in the activation of these channels. These include protein kinases, guanine nucleotides (which are involved in glucose sensing) and regulation by the neurotransmitters dopamine, serotonin and acetylcholine, in addition to the regulation of these channels by voltage (Perez-Reyes, 2003). These pathways may be pathways involved in leucine sensing, leading to activation or inhibition of these channels.

3.5.3 A subpopulation of leucine sensing cells are dopaminergic

Studies in chapter 2 identified POMC and NPY neurons were able to sense leucine. 25% of POMC neurons, and slightly fewer NPY neurons, are activated or inhibited by leucine. Some POMC neurons were leucine inhibited, and some NPY neurons were leucine excited, in contrast to a classical understanding of their function, but in line with a recent report showing that 25% of POMC GFP neurons expressed AgRP/NPY (Lam et al., 2017). This was also seen in our data, with one of the POMC GFP leucine activated neurons actually expressing higher levels of AgRP/NPY.

Characterisation of the remaining cells was challenging, as expression of NPY, Vasopressin, and other known neurotransmitters, was high in these other cells. Without having a known population of these neurons, as for the POMC and NPY GFP neurons to compare to, it is hard to identify what expression level is sufficient to classify a neuron into a certain category.

7 of the leucine sensing neurons were TH positive, suggesting that leucine bidirectionally regulates the activity of this known population of arcuate nucleus neurons. A subpopulation of these TH neurons, along with POMC neurons, have previously been shown to express T type calcium channels, suggesting a link between these cells and this mechanism (Qiu et al., 2006; Zhang and van den Pol, 2015). Dopaminergic neurons were split into two subpopulations, one identified as expressing the T Type channel and having burst like firing, the second not expressing the channel and not having this electrophysiological property (Zhang and van den Pol, 2015). These TH positive neurons were GABAergic, and had an orexigenic effect when studied using optogenetics (Zhang and van den Pol, 2016). The neurons project to AgRP/NPY and POMC neurons in the Arcuate nucleus, in addition to other brain regions.

Of the 8 leucine sensing cells that expressed a T Type calcium channel, 2/4 of the leucine activated and 1/4 of the leucine inhibited cells were TH positive. Given that pharmacological blockade of T Type channels only halved the percentage of leucine sensing cells, a different

pathway may be necessary for leucine sensing in these TH neurons. It suggests leucine sensing TH neurons do not necessarily overlap directly onto either the T or non T type expressing TH populations.

Another interesting finding having analysed the heatmaps was that two pairs of cells appeared to show similar gene expression patterns. Act 7 and Inh 0, and NPY Inh 0 and NPY Con 1 showed near identical gene expression patterns. Act 7 and Inh 0 are both TH positive cells. Further analysis performed by Brian Lam (not shown) showed they were highly similar, but there were subtle differences in gene expression between them. Given that these cells show different leucine responses, it will be important to determine the subtle differences that cause them to respond differently.

3.5.4 Phospho TRAP hits in leucine sensing single cells

I examined if the hits identified in the Phospho TRAP study were expressed by the single leucine sensing cells. No specific gene was noticeably enriched in the leucine sensing cells. There are several possible explanations for this.

Although hits in the phospho TRAP study are enriched in leucine sensing neurons, they may also be expressed in non-leucine sensing cells. The expression of some genes alone may not be sufficient for leucine sensing, but a combination or set of genes in a pathway may all need to be expressed for a cell to sense leucine. A protein may be expressed in many cells, but is only used for leucine sensing in a subpopulation of cells. As seen in chapter 2, many pathways known to be involved in leucine sensing *in vivo* were not necessary for the rapid calcium response to leucine. Additionally, as the timescale of the responses is different, it may not be surprising that the pathways identified in the phospho TRAP study do not overlap with the single leucine sensing cells.

3.5.5 Drawbacks of single cell sequencing study

The single cell sequencing data set, along with the phospho TRAP data set, provide exciting insights into the mechanisms by which leucine is sensed. The single cell sequencing data set does have some drawbacks in hunting for markers of leucine sensing cells. Leucine treatment could have caused changes in gene expression in the single cells, as capture of these neurons happened at least 20 minutes, and up to 90 minutes, after leucine application. Many signalling pathways downregulate gene expression in response to strong pathway activation, which could result in leucine sensing proteins not being identified in the

single cells (Cheung et al., 2012; Vogel and Marcotte, 2012). Stress due to the culture protocol could desensitise cells to leucine, resulting in false negatives. Consequently, this may not be as useful a tool to identify the mechanisms by which leucine is sensed. Some cells are activated by saline application alone (chapter 2). This means some cells may not be responding to leucine, but are randomly activated (false positives). It is hard to quantify this number, and only a data set of sufficient size could reduce the effect size.

Another challenge with the data is that some genes, such as those coding for calcium channels in the intracellular calcium stores (Figure 3-10) are expressed by few neurons. These genes should be expressed in most cells, as most cells, including neurons, have intracellular calcium stores regulated by these receptors (Lu and Fivaz, 2016). This could be a result of the culture itself, where gene expression may be downregulated due to the stress of the extraction and placing the cells in media that is not ideal for their survival outside the brain (for example, changing GK expression in neuron cultures in (Kang et al., 2006)). Given that the neurons structurally are changed, having had axons and dendrites sheared in the extraction, genes may no longer be expressed until the neurons have reformed those projections. For instance, synaptic proteins may no longer be expressed normally (Lesuisse and Martin, 2002), and the usual structure of intracellular calcium stores may be altered, meaning the necessary receptors for signalling are not required as the usual synaptic inputs are absent.

Another explanation for the absence of gene expression of intracellular store calcium channels could be the turnover rate of these proteins. The turnover rate of IP₃R measured in unstimulated cell lines is low, with a receptor half-life of 10 – 20 hours (Khan and Joseph, 2003; Wojcikiewicz, 2012). This means gene expression does not need to occur at high levels to maintain expression of functional protein, although mRNA expression was not quantified in most of the studies referred to in Wojcikiewicz, 2012.

The detection limit of the RNA sequencing may also prevent detection of lowly expressed genes, although the sequencing performed is of higher depth than for other techniques presently used in DropSeq single cell sequencing studies.

There is a high degree of heterogeneity within the cells collected. There are at least three neurochemically defined populations (TH, POMC and NPY) of cells sensing leucine, with others as yet undefined. In addition, 3 of the leucine sensing cells may be non-neuronal. Our

control pool of cells is less heterogeneous, making comparisons challenging. Consequently, we decided not to perform a more thorough analysis of the data set, looking at genes uniquely expressed in the leucine sensing neurons, until we capture more neurons. We hope to focus on POMC neurons only, that are activated or inhibited by leucine, to reduce heterogeneity in the analysis, meaning the true markers of leucine sensing cells can be more easily identified. This approach is challenging as some of the captured POMC neurons are not true POMC cells, instead being AgRP/NPY positive. This means we need to capture more cells so we have a true POMC population, to reduce heterogeneity.

Leucine responses are variable, so to aid with identification of markers of leucine sensing cells, we subdivided the leucine sensing population based on the response profiles. The differences in leucine response profiles was not explored in greater depth in chapter 2. The 3 categories of responses for both activated and inhibited neurons may reflect an additional degree of heterogeneity in the mechanisms by which leucine is sensed, and may require a higher number of neurons to be captured to perform a thorough analysis.

3.5.6 Are glucose and leucine sensed by the same cells?

As discussed in the Chapter 1, understanding if nutrient sensing occurs in the same or different cells will help understand how the hypothalamus is organised to control energy homeostasis. Although the number of single hypothalamic neurons sequenced here is a small fraction of the neurons expressed in the hypothalamus, it provides unique insights into this question. As there is no known marker for leucine sensing neurons at present, the one of the only ways these neurons can be identified is by observing them respond to leucine, as has been done in these studies, using calcium imaging. The expression of known markers of glucose sensing neurons can be examined in these cells to see if leucine sensing neurons may also be glucose sensing.

Of the glucose sensing genes assessed, *Gck*, *Slc2a3* and *Gucy1b3* were most widely expressed in the cells. However, there was no noticeable enrichment of any of these genes in leucine sensing neurons. The classical markers of the pancreatic beta cell glucose sensing mechanism were not all expressed in one neuron. This is consistent with previous studies showing expression of some glucose sensing proteins in non-glucose sensing neurons (Kang et al., 2004; Lynch et al., 2000; Sanz et al., 2011). This data shows that there may be some overlap between glucose and leucine sensing cells. It suggests that for glucose and leucine,

some cells sense just glucose, some just leucine, and some cells can sense both, as for fatty acids and glucose (Le Foll et al., 2009). The control population is enriched for POMC and AgRP/NPY neurons, as stated before, so may not serve as a good control population to make this assessment.

The overlap between amino acid and fatty acid sensing cells was also examined, but two important candidate genes identified in mediating fatty acid sensing, *Cd36* (Le Foll et al., 2009, 2013), and *Cpt1a* (Pocai et al., 2006), were not expressed in any of the MBH neurons. Other fatty acid sensing candidate genes were not noticeably enriched in this small population of leucine sensing neurons sequenced.

The concept of “metabolic sensing neurons”, responsive to glucose, amino acids and fatty acids was also explored (Levin, 2002, 2006; Levin et al., 2011), with two leucine sensing neurons expressing *Gck* and *Fasn* (fatty acid synthase), but these genes are not markers for all glucose and fatty acid sensing neurons, meaning more neurons may be “metabolic sensors”. Functional analysis of neuronal activity in response to leucine, glucose and oleic acid concentration coupled with assessing gene expression may be a more reliable guide to assess the metabolic sensing neuron concept. However, as discussed earlier, the cells that respond to leucine in calcium imaging experiments are unlikely to represent the whole leucine sensing population, so it is not possible to comment further on the metabolic sensing neuron hypothesis.

3.5.7 Further studies

qPCR on the genes identified in Table 3-1 is being performed, to confirm that the hits identified are genuine. If time allowed, performing a second identical phospho TRAP experiment to generate independent samples would strengthen this. The final step once potential targets have been identified is to test if these hits have an effect on leucine sensing *in vivo*.

The promising results with the T Type calcium channel require further confirmation. For instance, in brain sections of MBH leucine treated mice, do cells expressing cFOS also express the calcium channel?

The dopaminergic neuronal population could be studied further, for instance, by performing similar studies on a TH reporter line, as for NPY and POMC GFP mice, to determine what percentage of TH neurons are leucine sensing.

If novel sensing mechanisms are identified, and pharmacological inhibitors were not available, transfection of neurons *in vivo* or *ex vivo* could be conducted, with calcium imaging used to test if the responses were abolished. Transfection of primary neuron cultures is challenging, but possible (Kang et al., 2006).

4 Chapter 4: Assessing the role of AgRP Neurons in the control of Glucose Homeostasis

4.1 Chapter Summary

AgRP Neurons are essential for feeding, and have been shown to play roles in controlling glucose homeostasis, regulating hepatic glucose production, and brown adipose tissue and skeletal muscle glucose uptake. Given that these neurons are glucose inhibited, and shown to play a role in controlling glucagon secretion, I sought to explore if these neurons are involved in regulating the counter regulatory response (CRR) to hypoglycaemia. Using chemogenetic tools, I examined the effect of activating AgRP neurons on glucose homeostasis and on the CRR. In contrast to a recent chemogenetic study, activation of AgRP neurons during an insulin tolerance test did not cause a significant change in blood glucose concentrations. This is in contrast to a recent study where blood glucose concentrations were shown to increase upon AgRP neuron activation. Unexpectedly, significantly lower blood glucose concentrations were measured when AgRP neurons were activated after a 2 hr fast. No significant difference in blood glucose concentrations was measured in a glucose tolerance test conducted immediately after this fasting study. AgRP neuron activation during a hypoglycaemic clamp did not change the glucose infusion rate required to maintain hypoglycaemia, and did not cause changes in plasma glucagon concentrations in response to hypoglycaemia. This study suggests AgRP neurons may not play a role in the CRR, although the clamp studies were performed on a small number of animals which require further confirmation.

4.2 Introduction

4.2.1 Refining our understanding of the role of AgRP neurons in feeding behaviour

AgRP neurons are a neuronal population involved in feeding behaviour. A study using diphtheria toxin to ablate AgRP neurons in adult mice demonstrated that without these neurons, animals starved (Gropp et al., 2005; Luquet et al., 2005). Optogenetic activation of just 800 AgRP neurons generates a maximal feeding response (Aponte et al., 2011). Recent work has refined our understanding of how these neurons control feeding behaviour, and how they interact with other feeding neurocircuits. AgRP neurons project to multiple sites within the brain, but each AgRP neuron projects to a single site, a one – one system (Betley et al., 2013). Using optogenetics, the Sternson lab demonstrated that activation of some AgRP subpopulations, based on the sites they project to, did not elicit a feeding response

(Betley et al., 2013). This demonstrates that different projections control different functions. The ability to sense nutritional information varies among AgRP neurons. Only AgRP neurons projecting to extra - hypothalamic sites expressed the leptin receptor (Betley et al., 2013). In contrast, ghrelin receptor expression was not found in a specific population of AgRP neurons. This data suggests AgRP neurons are heterogeneous, both in terms of the functions they control and their ability to respond to an animal's nutritional state.

Using *in vivo* calcium imaging, it has been shown that AgRP neuron activity rapidly falls when mice are exposed to the sight or smell of food (Betley et al., 2015; Chen et al., 2015); the authors of these studies concluded that AgRP neurons are principally involved in food seeking behaviour, but not food consumption (Sternson and Eiselt, 2017). This finding is masked in optogenetic or chemogenetic studies, where the activity of AgRP neurons cannot be suppressed. In addition, it has been proposed that AgRP neuron activity serves as a negative valence signal, so that over time animals learn to avoid cues that will not lead to food consumption, and consequent restoration of homeostasis (Betley et al., 2015; Sternson and Eiselt, 2017)

4.2.2 Roles of AgRP Neurons in the control of glucose homeostasis

Besides food seeking behaviour, which has been the major focus of studies of AgRP neurons, these neurons play other roles, such as in the regulation of whole body glucose homeostasis. Table 4.1 lists publications where AgRP/NPY neurons have been shown to control glucose homeostasis, at three principal levels, the control of hepatic glucose production (HGP), skeletal muscle glucose uptake and BAT glucose uptake, the latter two via the modulation of insulin action on these tissues.

Recent work from the Bruning lab demonstrated that activation of AgRP neurons using both chemo- and optogenetics led to an increase in blood glucose concentrations, due to increased BAT insulin resistance, and subsequent reduction in glucose uptake (Steculorum et al., 2016). No increase in HGP was observed when AgRP neurons were chemogenetically activated during euglycaemic hyperinsulinemic clamps (Steculorum et al., 2016). An increase would be expected based on previous findings from this group where knock out of insulin receptors (IR) from AgRP neurons prevented insulin suppressing HGP (Könner et al., 2007). The authors propose this difference is due to MC4R dependent regulation of HGP that is not

observed in this acute activation study, that is revealed in germline genetic models (Steculorum et al., 2016).

Most studies showing a role for AgRP neurons in the control of HGP utilised a germline KO approach (see table 4.1). One important consideration is whether early manipulations of AgRP neurons leads to compensatory effects. When AgRP neurons are deleted using diphtheria toxin in the neonatal period, there is no starvation phenotype, as the neural circuits are able to rewire so the animals still eat in adulthood (Luquet et al., 2005). This suggests that using a germline approach to study AgRP neurons could alter the neurocircuits formed, meaning the phenotypes observed may not be relevant. In contrast to this hypothesis, a recent study using NPY-Cre as the driver to knock out the insulin receptor, showed no difference in phenotype between a germline and inducible knock out line (Loh et al., 2017). Although NPY is not expressed exclusively in AgRP neurons (Campbell et al., 2017), this work suggests that germline rewiring may not occur in this model, or may not impact the adult phenotype. This may depend though on the role of the protein knocked out in AgRP neurons during development of the AgRP neurocircuits.

Germline knock out of ribosomal protein S6K1 from AgRP neurons resulted in skeletal muscle insulin resistance, as observed in euglycaemic hyperinsulinemic clamps in overnight fasted mice (Smith et al., 2015). In this study, the authors analysed the neurophysiological properties of AgRP neurons using electrophysiology. They demonstrated that AgRP neurons were rendered dysfunctional, characterised by altered synaptic strength, changes to the resting spike firing frequency of the cells and an inability to sense changes in glucose concentrations. It is not clear from their study what percentage of neurons S6K1 is expressed in, although another study suggests pS6K1 was found in 90% of AgRP/NPY neurons (Cota et al., 2006). This suggests during normal neuronal function, AgRP neurons are necessary for skeletal muscle to be insulin sensitive. If true, then using chemogenetic tools to probe this aspect of AgRP neuron function, I would expect to see a reduction in blood glucose levels upon AgRP neuron activation, if the other effects on blood glucose concentrations that AgRP neurons can cause were not occurring.

A recent study stands in contrast to these findings. In adult mice, IR was knocked out from NPY neurons (Loh et al., 2017). IR knock out would be expected to remove the inhibitory effect of insulin from AgRP/NPY neurons, increasing action potential firing of AgRP neurons,

suggesting they are over activated in this model. These mice also developed skeletal muscle insulin resistance (Loh et al., 2017), in contrast to Smith et al., 2015 where less active AgRP neurons caused the same phenotype.

Another parameter AgRP neurons may modulate is called glucose effectiveness, defined as “The efficiency by which glucose can restore its own concentration independent of any dynamic insulin response” (Bergman, 1989). In rodents, glucose effectiveness is determined by applying the minimal model methodology to results of frequently sampled IVGTTs (FSIVGTT) (Alonso et al., 2012; Burke et al., 2017b; Morton et al., 2013). Relatively little is known about how the brain controls this process (Schwartz et al., 2013), but a recent study suggests POMC and AgRP neurons may be involved (Chhabra et al., 2016). i.c.v. AgRP infusion increased glycosuria, improving glucose tolerance (Chhabra et al., 2016), although the reason for increasing glucose excretion in the fasted state is not clear.

4.2.3 Work in this chapter and hypotheses to test

The literature suggests AgRP neurons can affect glucose homeostasis at multiple levels. Given that some AgRP neurons are glucose inhibited (Fioramonti et al., 2007; Hao et al., 2016; Smith et al., 2015), and AgRP neurons have been shown to play a role in mediating glucagon secretion (Wang et al., 2014), I sought to explore if AgRP neurons played a role in controlling the CRR to hypoglycaemia. The role AgRP neurons play in the control of glucose homeostasis at non – hypoglycaemic glucose levels were also explored. I undertook a chemogenetic study to explore whether activating these neurons during hypoglycaemia or during glucose and insulin tolerance tests altered glucose homeostasis.

I hypothesise that:

- 1) Activation of AgRP neurons will lessen the severity of hypoglycaemia, through an increased CRR, mediated via increased glucagon secretion.
- 2) Activating AgRP neurons at euglycaemia will increase blood glucose concentrations, as seen by Steculorum et al., 2016

Table 4-1: Studies examining the role of AgRP Neurons in the control of glucose homeostasis

Paper	Model	Effect Seen	Study details	Caveats
AgRP Neurons Increase Hepatic Glucose Production				
(Wang et al., 2014)	Ghrelin receptor (GHSR) restored in AgRP Neurons in GHSR KO Mice	Ability of ghrelin to ↑HGP restored. Glucagon secretion restored	Males Beyond 5 – 7 weeks old 16hr fasting glucose	Receptor not restored in all AgRP neurons Effect on circuits of GHSR KO
(Könner et al., 2007)	AgRP Neuron IR KO Mice	Insulin cannot ↓HGP Normal baseline HGP No GTT or fasting glucose effect	Euglycaemic hyperinsulinemic clamp, 16 hr fast Gender not reported in clamps 15 weeks old	Germline KO
(Lin et al., 2010)	AgRP Neuron IR Restoral in IR KO Mice	Insulin ability to ↓HGP restored	Males, 3-5hr fast, Euglycaemic hyperinsulinemic clamps 14 – 16 weeks old	Germline KO
(Ruan et al., 2014)	AgRP Neuron O-GlcNAc transferase KO	↓gluconeogenesis No change in insulin sensitivity Protects against HFD induced insulin resistance ↓spontaneous firing rate ↓outward K ⁺ current Similar membrane potential Improved glucose tolerance	GTT or PTT, 5 month old females, overnight fast ITT, 6 hour fast, 5 month old females HFD ITT in 5 month old males	Germline KO
(Ren et al., 2012)	AgRP Neuron FoxO1 KO	↓HGP during PTT and clamps	Euglycaemic hyperinsulinemic clamps, fast overnight (?)	Germline KO

			PTT, overnight fast Gender and age not reported	
(Kuperman et al., 2016)	AgRP Neuron CRFR1 KO	↓HGP during PTT (females only) No effect in GTT, ITT, (both genders)	PTT, overnight fast ITT, GTT, 5.5 hr fast Age not reported	Germline KO
(Ren et al., 2015)	AgRP Neuron Gpr17 KO	↓HGP (PTT) Improved glucose tolerance (GTT)	16 week old male mice PTT – 5 – 6hr fast GTT – overnight fast	Germline KO
AgRP Neurons Cause Insulin Resistance in BAT				
(Steculorum et al., 2016)	AgRP DREADD (germline)	Insulin resistance in BAT Increased circulating insulin, trend towards increased glucagon (both in non-clamp conditions)	Gender and age not reported GTT – 16hr fast ITT – no fast before study Clamps (Euglycaemic hyperinsulinemic, 4 hr fast)	Only 60% of neurons express receptor
	AgRP Optogenetics (unilateral injection)	Insulin resistance in BAT No effect on glucose tolerance	GTT and ITT, as above	Unilateral injection
AgRP neurons and muscle insulin resistance				
(Smith et al., 2015)	AgRP S6K1 Germline KO	Skeletal muscle insulin resistance Altered synaptic strength and glucose sensing AgRP Neurons “Non-functional”	Euglycaemic hyperinsulinemic clamps, Males, fast overnight Age not reported	Germline KO
(Loh et al., 2017)	NPY Neuron IR KO (inducible and germline)	↓HGP at baseline HGP still suppressed in clamps ↑fasting blood glucose	Euglycaemic hyperinsulinemic clamps, 5hr fast ITT or GTT, fast 4 or 6 hours	NPY expressed in other brain regions

		Insulin resistance in muscle	Males, 10 – 22 weeks old	NPY neurons in ARC do not all express AgRP
AgRP Neuron Perturbations with no attributable effect				
(Tsaousidou et al., 2014)	AgRP Neuron IKK2 (inhibitor of nuclear factor kappa-B kinase 2) KO	↑AgRP Neuron firing Insulin signalling via AgRP neurons blocked, glucose intolerant	ITT, 10 weeks, Males GTT, 6hr fast, 11 weeks, Males	Germline KO
(Vernia et al., 2016)	AgRP cJun NH ₂ -terminal kinase KO	Impaired glucose tolerance on HFD, increased fasting blood glucose	GTT, 16 weeks old, Males, Overnight fast Age not reported	Germline KO
(Joly-Amado et al., 2012)	AgRP Neuron Neonatal Ablation	Improved glucose tolerance in ablated mice on HFD	OGTT, 5 hr fast, Males, after 6 month HFD	Ablation model, circuits can rewire in development
(Zhang et al., 2008)	AgRP Neuron IKKβ/NF-κβ KO	Improved glucose tolerance on HFD vs controls	GTT, Overnight Fast 12 weeks HFD from weaning Gender not reported	Germline KO

4.3 Materials and Methods

4.3.1 Animals and Stereotactic Surgery

All mice were housed under controlled temperatures (21–23°C) and a constant 12 hr light/dark schedule and fed ad libitum. This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

8 – 14 week old AgRP-Ires-Cre Mice (Jackson Lab # 012899) underwent stereotactic surgery to allow a bilateral injection of AAV8-hSyn-DIO-rM4Dq-mCherry (UNC Vector Core) at coordinates -1.1mm Anterior/Posterior, -5.9mm Dorsal/Ventral from bregma. The cannula tips were spaced 0.8mm apart, allowing injection 0.4mm either side of the midline. 300nl of virus was injected per side, at a rate of 75nl/min. Injector was held in place for 6 – 12 minutes post injection. Studies began 2 weeks after surgery.

4.3.2 Feeding Studies

Mice were singly housed for 2 hours, from 9am. CNO, the agonist for the GPCR neurons were infected with, (Clozapine N-oxide, 1mg/kg, Sigma) or Saline was injected i.p. at 11am and food intake measured for 3 hours. A CNO 10x stock was prepared in advance (1mg in 1ml) before dilution to final concentration on the study day.

4.3.3 Glucose Tolerance Tests

Mice were fasted at 8am and brought into the study room to acclimatise. CNO (as above) or Saline was injected i.p. at 10am, and blood glucose measured at 15, 30, 60, 90 and 120 minutes. A glucose bolus (1g/kg i.p.) was given at 12pm and blood glucose measured at 15, 30, 45, 60 and 90 minutes post glucose injection (all glucose measurements performed with a One Touch Glucometer).

4.3.4 Insulin Tolerance Tests

Mice were fasted at 8am and brought into the study room to acclimatise. CNO or Saline was injected i.p. at 1.15pm, and mice given an i.p. dose of 1.5U/kg insulin (Actrapid) at 2pm. Blood glucose was measured before CNO injection (as above), and then at 0, 15, 30, 45, 60, 90, 120 and 150 minutes after insulin injection. If blood glucose dropped below 2.5mM, mice were given a glucose rescue and excluded from the study.

4.3.5 Vascular Surgery and Clamp Studies

Mice were studied 4 – 11 days after surgery to insert a jugular vein catheter. Mice were fasted on study day at 8am, brought into the study room to acclimatise and then placed in a restraint tube at least an hour before the clamp started at 2pm to allow attachment of the line for injection of insulin and dextrose. CNO (as above) or Saline was injected i.p. at 1.15pm.

Clamp studies consisted of a constant intravascular infusion of insulin at 20 mU/kg/min (Actrapid in 0.1% bovine serum albumin [BSA] in saline) with a 100 mU/kg initial priming bolus together with a variable infusion of 20% dextrose. Dextrose infusion rates were adjusted every 10 minutes to bring plasma glucose (measured through tail vein with One Touch glucometer) to, and maintain at, target level of 3 mM. Once plasma glucose level was below 4 mM, the clock was restarted and the animal was clamped for 60 minutes. At the end of the study a blood sample for glucagon measurements was collected via the infusion line in 1 mg/ml aprotinin in EDTA mixture, centrifuged at 13500 rpm for 90 seconds and plasma collected and stored. Analysis of blood samples was performed by Keith Burling, CBAL. The same mice were studied with both saline and CNO injections.

4.3.6 Immunohistochemistry

At the end of clamp study day 2, mice were euthanized by dislocation of the neck, and brains postfixed in 10% formalin. They were then submerged overnight in 20% sucrose in DEPC-PBS before sectioning. Using a freezing sliding microtome, brains were sectioned coronally into sections of 25 μ M thickness and collected free floating in PBS in 5 equal series. The sections were stored in antifreeze solution made up of 30% ethylene glycol and 20% glycerol in PBS solution, for later analysis.

Free floating tissue sections were washed six times for five minutes each (as for all washes) in PBS and pre-treated with 0.3% H₂O₂ for 30 minutes to inhibit endogenous peroxidases. The sections were further washed before blocking with 1% BSA in PBT (PBS with 0.04% Triton X-100) for one hour. This was followed by overnight incubation in rabbit anti-mCherry antibody (1:2000, Living Colours, ClonTech) at 4°C. The following day the tissue was washed before incubation with secondary Alexa Fluor 594 donkey anti-rabbit antibody (1:1000, Jackson Lab). The tissue sections were finally washed and then mounted onto Superfrost® Plus glass slides (VWR, UK), air-dried, and coverslipped with Vectashield mounting medium

(Vector Laboratories). Confocal microscopy was performed using a Zeiss LSM 510 Meta confocal microscope (images collected at 10x) using the Zen software package (Carl Zeiss Microscopy GmbH), to collect images of the MBH.

4.3.7 Statistics and Data Analysis

Insulin tolerance test was analysed using Two Way ANOVA. Glucose tolerance experiment was analysed in two halves by Two Way ANOVA (before and after glucose injection) with Bonferroni test to correct for multiple comparisons. % falls in glucose concentration after insulin injection were compared using a paired two tailed t – test. Due to low n values, statistics were not performed on clamp experiments. All statistics were performed using GraphPad Prism (Versions 6 and 7).

4.3.8 Supervision and study Assistance

I performed stereotactic surgery under supervision by Clémence Blouet. Glucose tolerance and insulin induced hypoglycaemia studies were performed with assistance from Chris Riches and Emmanuel Ogunnowo – Bada. Vascular surgery was performed by Emmanuel Ogunnowo – Bada. Hypoglycaemic Clamps were performed principally by Emmanuel Ogunnowo – Bada, during which Chris Riches and myself observed/learnt the technique.

4.4 Results

4.4.1 DREADD Receptor Expression in AgRP Neurons, and feeding data.

Given the role AgRP neurons play in glucose homeostasis, I set out to investigate what effect activating AgRP neurons would have on blood glucose concentrations. A chemogenetic approach previously used to study food intake, energy expenditure and locomotor activity (Krashes et al., 2011) was employed. This requires surgery on AgRP – Cre mice, to inject a Cre dependent AAV encoding a modified GPCR (with mCherry tag) that can be activated by a ligand called CNO (Clozapine N-oxide). There are no known endogenous agonists for these GPCR's (Alexander et al., 2009). Chemogenetic activation of AgRP neurons causes a rapid, sustained increase in food intake (Krashes et al., 2011). Animals can consume between 1.5 – 2g of food within 3 hours, during the light cycle, which is 5 – 10 times their normal food intake during this time period. Therefore, I used the presence of a feeding response to CNO as a positive control in animals before proceeding to examine glucose homeostasis, in addition to immunohistochemical confirmation post mortem.

Surgery was performed on 2 cohorts of animals, under the supervision of Clémence Blouet, but low numbers of the animals showed a feeding response upon injection of the ligand CNO. In a third cohort with 10 animals, all animals showed a feeding response to CNO injection (Figure 4-1A), suggesting AgRP neurons were being successfully activated. Total food intake ranged from 0.81 – 2.28g over 3 hours.

Expression of the virus in the brains of mice, as visualized using the mCherry reporter expressed by the virus (Figure 4-1B), was confirmed using immunohistochemistry. All the animals in this third cohort expressed mCherry in the arcuate nucleus. The exact number of AgRP neurons infected by the virus was not measured, due to time and equipment limitations when the study was conducted.

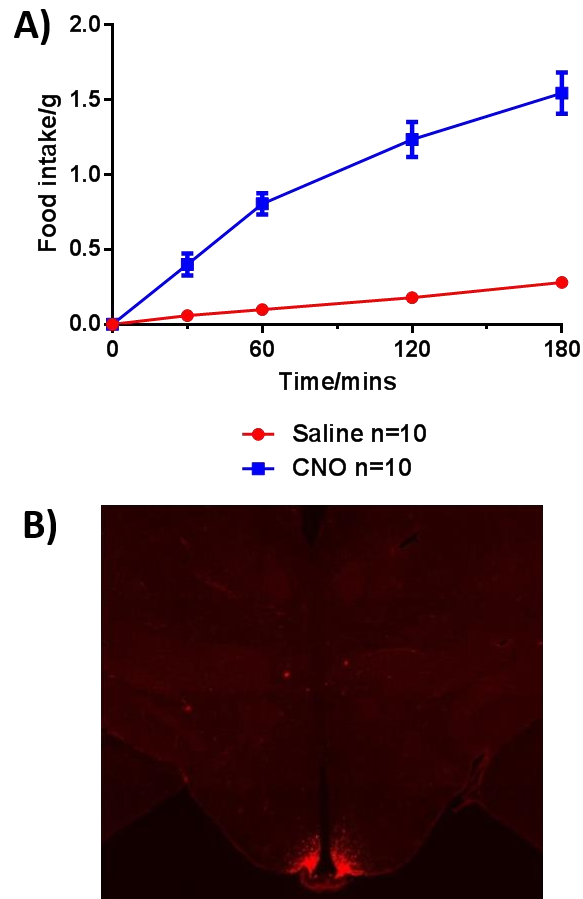


Figure 4-1: Confirmation of viral infection of AgRP neurons physiologically and immunohistochemically.

A) AgRP Neuron Activation using CNO leads to an increase in food intake, graph represents mean food intake \pm SEM, B) mCherry expression in the Arcuate Nucleus of AgRP Cre Mice injected with AAV expressing DREADD receptor.

4.4.2 The effect of activating AgRP Neurons on Glucose tolerance

Initially, in an attempt to assess the effect on the CRR to hypoglycaemia when AgRP neurons are activated, insulin tolerance tests with relatively high doses of insulin were performed. These are not classically reliable studies to assess counter regulation. During the studies, two animals became severely hypoglycaemic requiring a blood glucose rescue in one or both studies, so were excluded from the data set. There was no statistically significant difference (Figure 4-2A) in blood glucose concentrations between Saline and CNO treated mice during the study.

In an attempt to assess insulin sensitivity, I compared the percentage fall in blood glucose concentrations 45, 60 or 90 minutes after insulin injection, but there was no statistically significant difference between Saline and CNO treated groups.

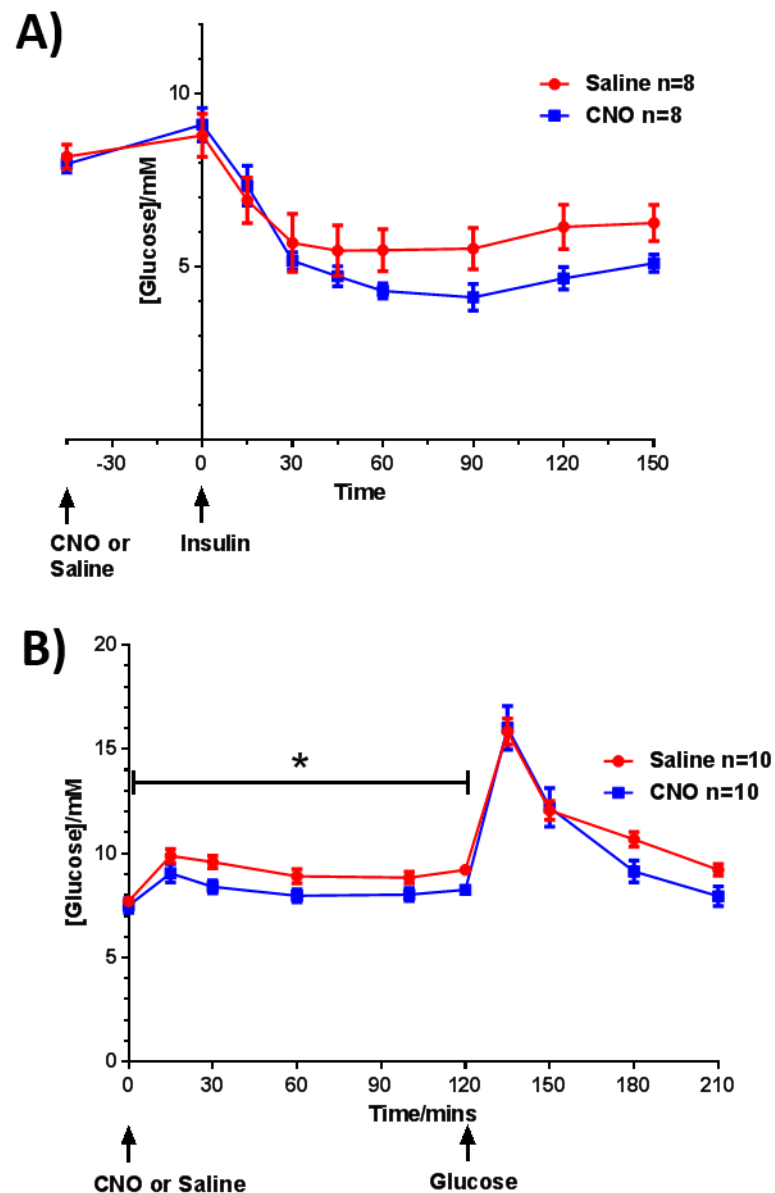


Figure 4-2: Effect of chemogenetic activation of AgRP neurons on blood glucose concentrations.

A) Activation of AgRP Neurons, using CNO, followed by Insulin injection, leads to lower blood glucose concentrations compared to saline control, $n=8$, crossover, 6 hr fast before 1.5U/kg insulin injection, B) AgRP Neuron Activation for 2 hours, after a 2 hour fast, followed 2 hours after by glucose injection (1g/kg). The two halves of the experiment were analysed separately by Two Way ANOVA, $*p<0.05$

Many mice did not reach hypoglycaemia making it difficult to draw conclusions about the role these neurons play in the CRR.

I also tested if activation of AgRP neurons could affect blood glucose levels, without injection of additional glucose or insulin. To do this, AgRP neurons were activated after a short fast. A 2 hour fast was chosen to minimise baseline activation of the neurons, which are known to be fasting activated (Hao et al., 2016; Takahashi and Cone, 2005). Within 15 minutes of CNO delivery, and lasting for the next two hours, there was a significant lowering of blood glucose levels (Figure 4-2B). After a glucose challenge, there was no statistically significant difference in blood glucose levels between Saline and CNO treated mice.

Immunohistochemistry showed that the DREADD receptor expression varied between animals, and feeding responses when AgRP neurons were activated were variable. I sought to test whether the glucose response observed correlated with the feeding response: if more AgRP neurons are activated, is the glucose response larger. For all studies, there was no statistically significant correlation between feeding response magnitude and glucose concentration difference (between CNO and saline treatment) (Figure 4-3).

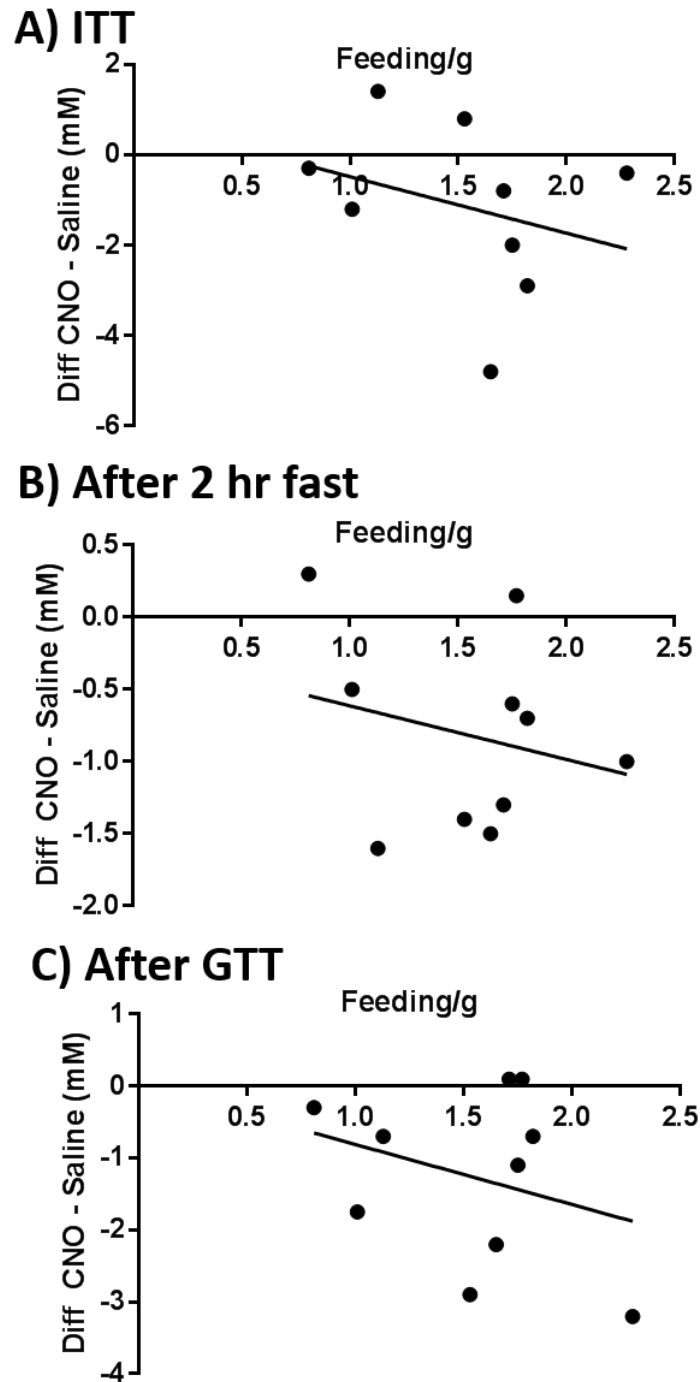


Figure 4-3: Correlation between magnitude of feeding response and blood glucose concentration change with chemogenetic activation of AgRP neurons.

Feeding response when AgRP neurons are activated plotted against difference in minimum blood glucose concentration when AgRP Neurons were activated, versus saline treatment. A) Difference during insulin tolerance test (one mouse excluded that received glucose rescue in both studies), $r^2=0.09$, B) Difference during 2 hour period after CNO/Saline injection, after 2 hour fast, $r^2=0.06$ and C) Difference after glucose tolerance test, $r^2=0.09$.

4.4.3 Assessing the effect of activating AgRP Neurons on hypoglycaemia CRR

Based on previous studies showing that some AgRP neurons are glucose inhibited (Fioramonti et al., 2007; Hao et al., 2016), I hypothesised that increasing the activation of these neurons during hypoglycaemia would increase the CRR, increasing blood glucose levels. Vascular surgery was performed to insert a jugular vein cannula to allow hyperinsulinemic hypoglycaemic clamps to be performed. While 9 of 10 mice survived surgery, only 4 mice survived the first study day to be studied with both CNO and saline, and so only the results from these mice are presented. Figure 4-4A shows the blood glucose levels measured during the study, demonstrating the induction of hypoglycaemia. We saw no significant difference in the dextrose infusion rate between groups (Figure 4-4B), suggesting there is no difference in the ability of mice to counter regulate in response to hypoglycaemia induction with CNO delivery. There was no significant difference in blood glucagon concentrations between saline and CNO treated mice at the end of the clamps (Figure 4-4C).

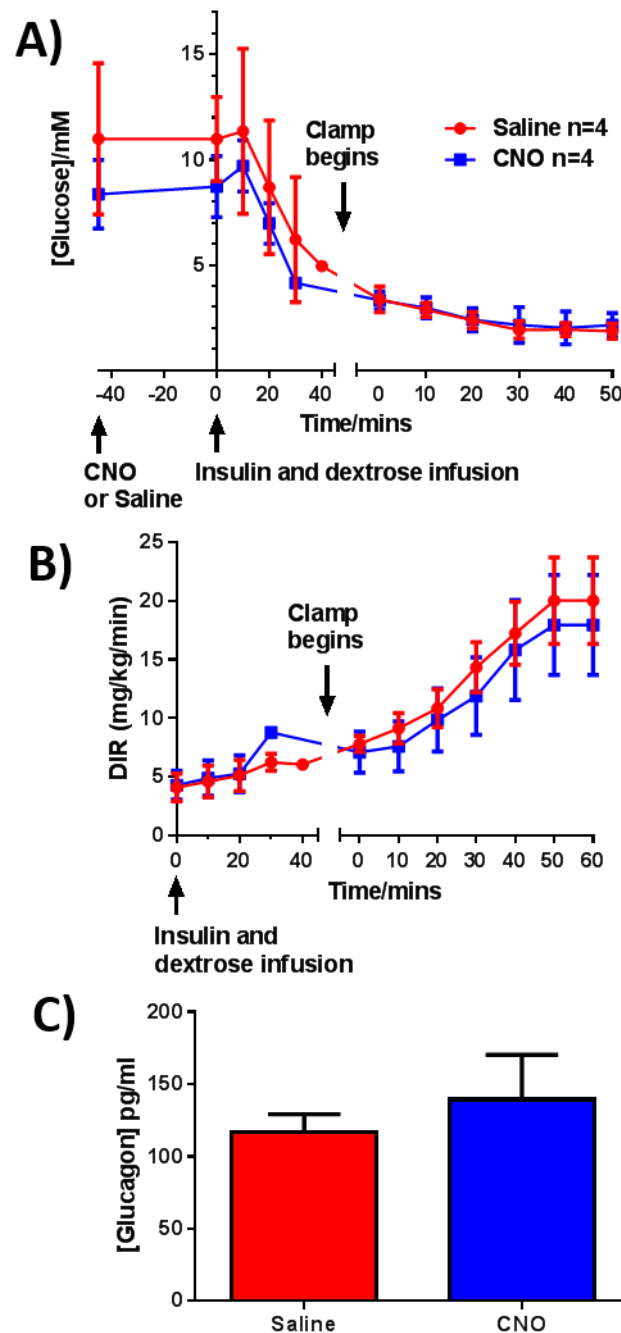


Figure 4-4: Chemogenetic activation of AgRP Neurons during Hypoglycaemic clamp study.

A) Tail vein glucose concentrations measured during hypoglycaemic clamp, which are used to determine dextrose infusion rate, B) Dextrose infusion rates. When animals reached hypoglycaemia (below 4mM), time was reset to zero, C) Plasma glucagon concentrations measured at end of the clamp (60 minutes), analysed by paired t -test. All bars in graphs represent mean \pm SEM.

4.5 Discussion

4.5.1 Key findings

Before starting the study, I hypothesised that activating AgRP neurons during hypoglycaemia would increase the CRR, increasing blood glucose levels in response to hypoglycaemia. Contrary to my initial hypothesis, activation of AgRP neurons during hyperinsulinemic hypoglycaemic clamps did not increase the CRR response to hypoglycaemia. In contrast to my second hypothesis and a recently published study (Steculorum et al., 2016), activation of AgRP neurons during an insulin tolerance did not increase blood glucose concentrations. Activation of AgRP neurons after a 2 hour fast decreased blood glucose concentrations, but this effect did not persist during a glucose tolerance test.

4.5.2 Differences compared to previously published studies

The designs of my studies compared to those published were different. I conducted my studies before Steculorum et al., 2016 was published, but would have sought to replicate their study design had I performed my studies after theirs. Key differences which could account for my inability to replicate their results in glucose and insulin tolerance tests are discussed below.

4.5.2.1 Different AgRP neurons expressing DREADD receptor

DREADD receptors were expressed in AgRP neurons using a germline approach in Steculorum et al., 2016, whereas I infected neurons using virus injection into the brain of AgRP – Cre mice. This may be a key source of variation. Stereotactic delivery of AAV is highly unlikely to fully infect all AgRP neurons. However, neither did the germline approach, where only two thirds of AgRP neurons expressed the DREADD receptor (Steculorum et al., 2016). In both studies, we may have targeted different AgRP subpopulations, which could account for the absence of an effect in my studies.

While the feeding responses I observed were within a similar range to previous studies (Krashes et al., 2011; Steculorum et al., 2016) it is possible that a larger number of neurons are required to be activated to increase blood glucose concentrations. Just 800 AgRP neurons, a small percentage of the total AgRP population, must be activated to generate a maximal feeding response (Betley et al., 2013).

At the time of conducting these experiments, the absence of a phenotype in the ITT's did not warrant further investigations, and other projects were consequently prioritised. This meant

a thorough analysis of the number and location of AgRP neurons infected with the DREADD receptor was not undertaken. Before certain conclusions, compared to Steculorum et al., 2016 are made, the number, location and projections of AgRP neurons infected will need to be determined. Studying the projections is challenging with the brain sections available, but some information could be determined based on expression of the leptin and ghrelin receptors (Betley et al., 2013). Additionally, confirming that only AgRP neurons have been infected by the virus will be necessary. mCherry was only expressed in the Arcuate nucleus, but this does not exclude the possibility that non – AgRP neurons were infected with the virus.

4.5.2.2 Different CNO injection patterns

CNO delivery varied between my study and Steculorum et al., 2016. Steculorum et al., 2016 used a lower CNO dose, but in their clamps regularly gave additional CNO injections to maintain neuronal activation. The effects of CNO have been shown to peak 1 hour post injection, with CNO concentrations significantly reduced at 2 hours post injection (Alexander et al., 2009; Guettier et al., 2009; Whissell et al., 2016). My inability to replicate their findings in the ITT may be due to the effects of CNO beginning to reduce at the point mice reached their nadir in the ITT (90 – 120 minutes after CNO injection). In the GTT, no difference in glucose concentration was observed after glucose injection, but this was over 2 hours after CNO injection, when it's effects may have begun to wear off. Co – injection of glucose and CNO would have overcome this problem.

4.5.2.3 Different insulin tolerance test designs

The design of the insulin tolerance tests I conducted was different to that of Steculorum et al., 2016. Steculorum et al., 2016 performed insulin tolerance tests, with 0 or 16 hour fasts, using both optogenetics and chemogenetics to activate AgRP neurons, and observed rising blood glucose concentrations in all their studies. This suggests the effect they observed is robust. I did not observe a difference in blood glucose concentrations in the ITT I conducted, with a 6 hour fast. It is noteworthy that in their ITT, CNO pre-treatment occurred 20 minutes before insulin injection, but blood glucose concentrations are presented relative to the initial baseline, so changes in blood glucose may have occurred before insulin injection that are not clearly presented.

4.5.3 Contrasting findings from 2hr fasting experiment

When AgRP neurons were activated after a 2hr fast, blood glucose concentrations were statistically significantly lower than when saline was injected. This stands in contrast to my other findings, where AgRP neuron activation did not cause statistically significant changes in blood glucose concentrations. This change in blood glucose concentration is in the opposite direction to those observed by Steculorum et al., 2016. The reasons for my inability to replicate the results of their study, discussed in section 4.5.2, may also serve as explanations for this result. The difference in lowest blood glucose concentrations was 0.8mM, and the CNO treated animals started 0.2mM lower than the saline treated animals.

Steculorum et al., 2016 did not assess the effect of activating AgRP neurons without exogenous application of glucose or insulin, so it is challenging to interpret the effects I saw. It is possible that different AgRP neuron subpopulations exist, that may control different aspects of glucose homeostasis. Steculorum et al., 2016 identified that AgRP neurons control BAT insulin resistance, and several studies have demonstrated a role for AgRP neurons in the control of hepatic glucose production, as outlined in section 4.2. One study identified a role for AgRP neurons in controlling skeletal muscle insulin sensitivity (Smith et al., 2015); if a subpopulation of AgRP neurons controlled this function, then activating them would be expected to reduce blood glucose concentrations, in line with the results I observed. However, as my results from one cohort of animals stand in contrast to the published results, and no other studies have shown a role for AgRP neurons in reducing blood glucose concentrations, further studies will be required before conclusions about different AgRP neuron subpopulations can be drawn.

In light of this data, a reanalysis of the expression of the virus would be extremely helpful in understanding which AgRP neurons were infected by the DREADD virus in my study compared to Steculorum et al., 2016.

4.5.4 The role of AgRP neurons in the control of the CRR to hypoglycaemia

It is possible that AgRP neurons play a role in the control of the CRR, but the study was not able to detect this effect. The insulin tolerance test is not principally designed to detect responses to hypoglycaemia, as it does not reliably cause mice to become hypoglycaemic, and is a better test for insulin sensitivity. The clamp studies lacked power as some mice were not clamped on the second study day.

In a ghrelin receptor knock out mouse, glucagon release was returned to normal levels when the ghrelin receptor was restored in AgRP neurons in adult mice, suggesting a subpopulation of AgRP neurons may be involved in the release of this counter regulatory hormone (Wang et al., 2014). A recent study using a PRV tracing virus has demonstrated that a multi synaptic pathway exists from the arcuate nucleus via the PVH and NTS to the pancreas, suggesting arcuate AgRP neurons could influence glucagon secretion (Rosario et al., 2016). In their chemogenetic study, Steculorum et al., 2016 saw a trend towards increased glucagon secretion one hour after AgRP neuron activation, although the effect was not statistically significant. Given that they used wild type, CNO injected mice as controls, instead of using the same animal as a control as I did (by running identical experiments with a saline injection), the power of the study is reduced and so may not have returned a statistically significant result with $n=10$.

Steculorum et al., 2016 also reported an increase in serum insulin concentration 1 hour after AgRP neuron activation, so it is possible that the reduction in blood glucose concentrations observed in our studies could be due to increased insulin secretion by AgRP neurons. However, the effects seen in Steculorum et al., 2016 are likely a secondary effect due to rising blood glucose concentrations with chemogenetic activation of AgRP neurons. Given that a pathway from AgRP neurons to the pancreas may exist (Rosario et al., 2016), AgRP neurons could directly regulate insulin secretion but as AgRP neurons are orexigenic, neuronal activation would be expected to reduce insulin secretion. Hyperglycaemic clamps to assess insulin secretion, in combination with chemogenetics, could be performed to assess this hypothesis.

This aspect of AgRP neuron function is also interesting in relation to the role of AgRP neurons in the clinical scenario of insulin induced hypoglycaemia, experienced by patients undergoing intensive insulin therapy to manage diabetes (McCrimmon, 2009; Ogunnowo-Bada et al., 2014). Patients may be hypoglycaemic, in the presence of high insulin concentrations due to their treatment regime. How the brain, and in particular AgRP neurons, process these competing signals to attempt to restore blood glucose concentrations to euglycaemia has not been explored. This may depend on whether a subpopulation of AgRP neurons is both insulin and glucose sensing, and what downstream effects this subpopulation has. Single cell RNA sequencing coupled with circuit mapping

techniques may allow the identification of these subpopulations, and their downstream targets.

4.5.5 Further Study Plans and questions to answer

It will be important to assess the expression of the mCherry reporter in the brains collected from the mice tested in the study presented in this chapter, to determine the number, location, and nature of the AgRP neurons targeted in this study, and compare this to the results from Steculorum et al., 2016.

I performed brain surgery on a fourth cohort of mice, but saw no feeding response in any of these animals, suggesting the surgery had been unsuccessful, so did not perform any further studies on this cohort.

If time had allowed, I would have performed a similar study but using an inhibitory DREADD, to suppress AgRP neuron activity, to see if blocking AgRP neuron activity during studies such as an ITT had the opposite effect to activating DREADD's on blood glucose levels.

Further studies should seek to address the effect of insulin induced hypoglycaemia on the activity of AgRP neurons.

5 Chapter 5: Assessing the role of glucokinase in AgRP neurons, in the control of glucose homeostasis and ingestive behaviour.

5.1 Chapter Summary

A subpopulation of AgRP neurons have been shown to be glucose inhibited, but the glucose sensing mechanism(s) in AgRP neurons are unclear. Glucokinase (GK), the enzyme involved in pancreatic glucose sensing has been shown to be involved in brain glucose sensing and is expressed in AgRP neurons. Our lab previously generated a whole brain GK knock out mouse (GK KO). Male mice show an increased CRR to hypoglycaemia, are glucose intolerant at 6 – 10 weeks old, and show an increased glucose preference in two bottle preference studies versus fructose or sucralose. Our lab went on to generate an AgRP neuron specific GK KO mouse. I phenotyped these animals and their litter mate controls, performing feeding studies, glucose, insulin and pyruvate tolerance tests, at 6 – 10 and 18 – 25 weeks old in both genders, and glucose preference studies on 6 – 10 week old male mice. Preliminary results suggest AgRP GK KO female mice may have altered glucose tolerance, but conclusions can only be drawn once further mice have been phenotyped, and the success of the glucokinase knock out from AgRP neurons has been confirmed.

5.2 Introduction

5.2.1 Glucokinase in the brain and pancreas

The brain principally utilises glucose as a fuel for cellular processes, so it is essential that it is able to sense changes in glucose concentration, to be able to ensure sufficient glucose supply.

Glucokinase is one of a family of enzymes called hexokinases, which phosphorylate glucose to form glucose – 6 – phosphate. Unlike the high affinity hexokinases, the activity of which saturates at low glucose concentrations, glucokinase has a low affinity for glucose, so glucokinase activity does not saturate as glucose concentrations increase (Matschinsky, 2009). This makes it ideally suited to act as a “glucose sensor” (Ogunnowo-Bada et al., 2014). Glucokinase, expressed in the pancreas, is critical for glucose stimulated insulin secretion. Mutations in the *Gck* gene cause diabetes of varying severity depending on the mutation and the number of mutant alleles (Njølstad et al., 2003; Osbak et al., 2009), demonstrating the essential glucose sensing role GK plays. The pancreatic beta cell glucose sensing mechanism has served as a model for a proposed glucose sensing mechanism in the brain, involving

glucose transporters at the cell membrane, and the K_{ATP} channel downstream of GK, that transduces changes in cellular ATP concentration to changes in membrane potential. A number of studies have demonstrated overlapping expression of the proteins necessary for this mechanism in hypothalamic neurons (Dunn-Meynell et al., 2002; Kang et al., 2004; Miki et al., 2001). However, not all glucose sensitive neurons express these proteins, and some are expressed in non – glucose sensing neurons (Kang et al., 2004; Lynch et al., 2000; Sanz et al., 2011).

Previous studies in our lab have explored the contribution of brain GK in controlling glucose homeostasis. I.c.v. infusion of glucose at the start of an ivGTT increased early phase glucose stimulated insulin secretion from the pancreas (Osundiji et al., 2012). Using the same study design, insulin secretion was reduced when GK inhibitors were infused (Osundiji et al., 2012). Taken together, these experiments demonstrate that brain glucose sensing via glucokinase plays a role in regulating insulin secretion in response to an i.v. glucose challenge.

Separately, i.c.v. infusion of a GK inhibitor increased protective glucoprivic feeding (Osundiji et al., 2010), showing that glucose sensing via brain GK plays a role in the initiation of glucoprivic feeding. In another study, i.c.v. infusion of a GK inhibitor increased cFOS expression in AgRP/NPY neurons (Zhou et al., 2011), suggesting these neurons sense glucoprivation, or form part of a neurocircuit that responds to glucoprivation. Projections from glucose responsive hindbrain catecholaminergic neurons, which are necessary for glucoprivic feeding (Hudson and Ritter, 2004; Ritter et al., 2000, 2001, 2011), can change AgRP and NPY gene expression (Khan et al., 2014). This data demonstrates glucose sensing via GK is important for initiating glucoprivic feeding, and may mediate these effects via AgRP/NPY neurons.

Using a model where AgRP neurons were deleted during development, there was no difference in glucoprivic feeding in adult mice compared to mice with intact AgRP neurons (Luquet et al., 2007). This suggests AgRP neurons are not the site of hypoglycaemia detection to generate a feeding response, and are not part of the neurocircuitry responsible for glucoprivic feeding. However, in this model, compensatory changes could occur during development to form alternative circuits to allow glucoprivic feeding to occur (Luquet et al., 2007), so a role for AgRP/NPY neurons in glucoprivic feeding (as a primary sensing site or

part of the neurocircuit for responding to glucoprivation) cannot be ruled out from this study alone.

5.2.2 Can brain glucokinase act as a glucose sensor?

Any proposed glucose sensor must be able to respond to physiologically relevant concentrations of glucose, but there has been debate about the precise concentration range that neurons in the brain are exposed to. Early studies using glucose microelectrodes suggested brain glucose concentrations in rats ranged from 0.5 – 4.5mM glucose, 15 – 30% of the concentration found in the blood (Silver and Erecińska, 1994), a finding subsequently extended to the hypothalamus (Silver and Erecińska, 1998). Later studies using microdialysis have shown that glucose concentrations in the VMH can fall to 0.3mM (Dunn-Meynell et al., 2009) during severe hypoglycaemia, and even lower during repeated bouts of insulin induced hypoglycaemia, below 15% of blood glucose concentrations (de Vries et al., 2003).

It has been hypothesised that due to the presence of a fenestrated blood brain barrier, neurons in the arcuate nucleus are exposed to higher glucose concentrations. A recent microdialysis study (Langlet et al., 2013), discussed at greater length in chapter 1, suggest falls in blood glucose concentrations during fasting cause plasticity in the blood brain barrier in the arcuate nucleus. Consequently, arcuate nucleus glucose concentrations paradoxically rose during a fast. Recent studies suggest that a small population of neurons in the arcuate nucleus, including AgRP/NPY neurons, may lie outside the blood brain barrier, and so may be exposed to larger changes in blood glucose concentrations (Olofsson et al., 2013; Yulyaningsih et al., 2017), although this requires further validation.

Glucokinase activity will only show small changes over the relatively narrow concentration range (0.5 – 4.5mM) found in the brain, particularly the small concentration change from euglycaemia to hypoglycaemia seen in the brain (Dunn-Meynell et al., 2009; Matschinsky, 2009; de Vries et al., 2003). This has led some to question how GK can serve as a glucose sensor, especially in GI (glucose inhibited) neurons (González et al., 2009). However, single neurons that were GI were shown to express glucokinase (Kang et al., 2004). Similarly, recent evidence showed that GK is enriched in GnRH neurons, which were demonstrated to be GI by electrophysiology, but, this does not prove that changes in glucose concentrations alter GK activity to regulate neuron firing (Stanley et al., 2013). Interestingly, when GK was deleted from SF1 neurons, electrophysiological analysis demonstrated SF1 neurons retained

glucose responsiveness (Steinbusch et al., 2016). However, SF1 GK KO did cause changes in body composition and CRR to hypoglycaemia, suggesting a dissociation between glucose responsiveness of neurons, and glucose induced changes in physiology (Steinbusch et al., 2016).

If glucokinase is not necessary for glucose sensing in neurons, then what role does it play? One hypothesis has suggested that GK is broadly expressed by hypothalamic neurons to increase their metabolic capacity, to support higher spontaneous firing activity seen in these neurons (Gonza et al., 2008). Deleting glucokinase from any population of neurons might render them defective, leading to the phenotypes observed as they no longer have sufficient energy to adequately support baseline functions. Functional studies are needed to confirm this hypothesis, but this is challenging given the lack of specific glucokinase inhibitors, which means this question of whether brain GK can act as a brain glucose sensor may remain unresolved for some time (Ogunnowo-Bada et al., 2014).

5.2.3 Glucose sensing mechanisms in AgRP neurons

AgRP neurons, or a subpopulation of these neurons, are involved in the control of whole body glucose homeostasis. A subpopulation of AgRP neurons are glucose inhibited, estimated to be between 25 and 40% (Fioramonti et al., 2007; Hao et al., 2016; Murphy et al., 2009a), but the precise mechanism by which they sense glucose is unclear. It is not clear if the subpopulation of AgRP neurons that are glucose sensitive are also those that control glucose homeostasis.

Table 5-1 summarises studies that have examined glucose sensing mechanisms in AgRP neurons. The majority of these studies are *ex vivo* analyses of AgRP/NPY neuron firing. While the figure of 25 - 40% of AgRP/NPY neurons being glucose sensitive is consistently reported, there are a number of candidate glucose sensing mechanisms. Two studies propose AMPK as the glucose sensor in AgRP/NPY neurons, but one of these showed that AgRP/NPY were glucose excited (Claret et al., 2007), and both used high glucose concentrations in the solution at some point during the experiment (Claret et al., 2007; Mountjoy et al., 2007). Some use high glucose concentrations briefly during extraction protocols to aid neuronal survival (Murphy et al., 2009a), but in these studies, cells were maintained in high glucose concentrations (glucose above 5mM, demonstrated to be the highest blood glucose

concentration in brain parenchyma, (Dunn-Meynell et al., 2009; Silver and Erecińska, 1994)) for longer time periods (Claret et al., 2007; Mountjoy et al., 2007).

Fioramonti et al., 2007 proposed, based on pharmacological evidence, that a CFTR like chloride channel is involved in AgRP/NPY glucose sensing, but more recent work from the same group (Hao et al., 2016) demonstrates that AgRP/NPY glucose sensing is dependent on an as yet undefined potassium channel, based on electrophysiological evidence. This is in contrast to other VMH GI neurons that they identified sensed glucose using an nNOS/AMPK dependent mechanism that may be linked to CFTR (Fioramonti et al., 2010, 2011; Murphy et al., 2009b). The authors also show that inhibition of AMPK in AgRP/NPY neurons (through injecting an inhibitor into the cytosol of neurons) does not stop AgRP/NPY neurons sensing glucose, demonstrating AMPK is not necessary for glucose sensing in AgRP/NPY neurons (Hao et al., 2016). The Sternson lab demonstrated that ghrelin acted via an AMPK dependent pathway in presynaptic neurons, to regulate the activity of AgRP neurons (Yang et al., 2011). Hao et al., 2016 developed these findings, showing that AMPK plays a role in fasting dependent regulation of AgRP/NPY neurons, but that this is AMPK in presynaptic neurons. This is consistent with another study showing AgRP/NPY neurons do not express nNOS (Leshan et al., 2012), another component of the proposed nNOS/AMPK/CFTR glucose sensing pathway.

Several of these studies used NPY GFP mice, or NPY specific probes (Fioramonti et al., 2007; Mountjoy et al., 2007; Muroya et al., 1999). In light of recent findings suggesting that a non AgRP expressing NPY population of neurons exists in the arcuate nucleus, these findings may be revised going forward (Campbell et al., 2017; Loh et al., 2017).

Using a novel glucokinase reporter line, Stanley et al., 2013 demonstrated that 30% of AgRP neurons express the enzyme GK, and functionally, 30% of AgRP/NPY neurons express cFOS after i.c.v. infusion of the GK inhibitor glucosamine (Zhou et al., 2011). In this later study, feeding was also increased, although this effect cannot be directly attributed to activation of AgRP neurons.

Table 5-1: Summary of glucose sensing mechanisms in AgRP Neurons

Paper	Model	Mechanism/Information
Non Mechanistic Studies		
(Muroya et al., 1999)	Rat hypothalamic neuron cultures (10 weeks old) Calcium Imaging	NPY positive neurons are glucose sensitive (10mM to 1mM)
(Murphy et al., 2009a)	Electrophysiology and secretion studies on NPY – GFP cells	40% NPY neurons glucose inhibited Fasting makes NPY GI neurons more glucose sensitive (2.5 – 0.3mM) Low glucose stimulates NPY release
nNOS/AMPK/CFTR		
(Claret et al., 2007)	AgRP Neuron AMPK α 2 subunit germline KO Electrophysiology	4/14 AgRP neurons in WT GE No GE AgRP neurons in KO 10, 2, 1, 0.1mM Glucose
(Mountjoy et al., 2007)	Calcium imaging of rat postnatal day 2 to 4 hypothalamic neurons with NPY specific probe	80% NPY neurons glucose inhibited, AMPK necessary (shown using Compound C and AICAR) 15 – 1mM glucose, high glucose media
(Fioramonti et al., 2007)	Electrophysiology of NPY GFP neurons	40% ARC NPY neurons GI Involvement of CFTR like Chloride channel 5 – 0.5mM glucose
Glucokinase (indirect evidence)		
(Wang et al., 2004)	Electrophysiology of medial ARC neurons (where NPY/AgRP neurons predominate)	Alloxan (GK inhibitor) modifies glucose sensing 2.5 – 0.1mM glucose
(Zhou et al., 2011)	i.c.v glucosamine, Rats	30% of AgRP/NPY neurons activated (cFOS), stimulates feeding
(Smith et al., 2015)	AgRP S6K1 Germline KO, Electrophysiology	Loss of ability to sense glucose (5mM to 1mM tested) Altered synaptic strength Lower resting spike firing frequency Reduced MBH GK and K ⁺ channel expression may mediate Skeletal muscle insulin resistance
(Hussain et al., 2015)	GK expression/activity changes in ARC of rats,	↑GK expression/activity, ↑glucose intake (converse shown)

	NPY secretion on brain sections from these rats	↑GK stops suppression of NPY release by glucose, 3 – 15mM Glucose K _{ATP} , P/Q Calcium channels involved
Other Mechanisms		
(Hao et al., 2016)	Electrophysiology of NPY GFP neurons	Over 40% of AgRP neurons GI Glucose sensing requires a K ⁺ channel nNOS and AMPK independent 2.5 – 0.5 or 0.1mM

5.2.4 Studies with whole brain glucokinase knock out mice

Emmanuel Ogunnowo – Bada, a previous PhD student and postdoc in the lab, phenotyped whole brain GK KO mouse line, generated by crossing a Nestin – Cre line to a GK floxed mouse. Male GK KO mice had a decreased dextrose infusion rate and an increased epinephrine response during hypoglycaemic clamps, suggesting they have an amplified CRR to hypoglycaemia. We believe this is because GK KO means the brain perceives blood glucose concentrations to be lower than they actually are during hypoglycaemia, increasing the CRR. Preliminary studies with only one control group suggested 6 – 10 week old brain GK KO mice are glucose intolerant. Brain GK KO mice have an increased glucose preference in two bottle choice preference studies, compared to control groups. However, the specific neuronal populations mediating these effects are not known.

5.2.5 Hypotheses to test

Given that AgRP neurons are GI and express GK, our lab generated an AgRP neuron specific GK KO mouse line, which I began phenotyping, as described in the rest of the chapter, to explore the role GK plays in AgRP neurons. I hypothesise that GK KO from AgRP neurons will result in over activation of these neurons, as the reduction in flux of glucose through this metabolic pathway will result in the neurons perceiving glucose concentrations to be lower than they are. Depending on the AgRP neuron subpopulation(s) GK is expressed in, I hypothesise that this will cause some or all of the following:

- Increase HGP
- Worsen glucose tolerance (and cause insulin resistance) if GK is expressed in the AgRP neuron subpopulation that regulates BAT glucose uptake

- Improve glucose tolerance (and increase insulin sensitivity) if GK is expressed in the AgRP neuron subpopulation that regulates skeletal muscle glucose uptake
- Increase feeding ad libitum, after a fast, and increase glucoprivic feeding

5.3 Materials and Methods

5.3.1 Animals

AgRP Glucokinase Knock Out Mice (AgRP GK KO) were generated by crossing AgRP-Ires-Cre Mice (Jackson Lab # 012899) with Glucokinase floxed mice (Postic et al., 1999). AgRP Cre^{+/-} Glucokinase^{fl/-} were crossed with AgRP Cre^{-/-} Glucokinase^{fl/-}, to generate AgRP Cre^{+/-} Glucokinase^{fl/fl}, and the control genotypes. Cre Control: AgRP Cre^{+/-} Glucokinase^{-/-}. Floxed Control: AgRP Cre^{-/-} Glucokinase^{fl/fl}. Wildtype (WT WT) AgRP Cre^{-/-} Glucokinase^{-/-}. Mice were housed under a standard 12 h light–dark cycle and were fed ad libitum. This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

5.3.2 Glucose Tolerance Tests

Mice were fasted at 8am and brought into the study room to acclimatise. Fasting blood glucose was measured between 1.30 and 2pm, before injection of a glucose bolus (1g/kg i.p.) at 2pm and measured again 15, 30, 45, 60 and 90 minutes after glucose injection. All glucose measurements were performed with a Glucomen Areo Glucometer (A Menarini Diagnostics). In some studies, blood glucose concentrations went higher than the maximum reading on the glucometer (33.3mM). These readings were logged as 33.3mM.

5.3.3 Pyruvate Tolerance Tests

Mice were fasted at 8am and brought into the study room to acclimatise. Baseline glucose readings were measured from 1.30 – 2 pm. A pyruvate bolus (1g/kg i.p.) was given at 2pm and blood glucose measured 15, 30, 45, 60 and 90 minutes post glucose injection. All glucose measurements were performed with a Glucomen Areo Glucometer (A Menarini Diagnostics).

5.3.4 Insulin Tolerance Tests

Mice were fasted at 8am and brought into the study room to acclimatise. Baseline glucose readings were measured from 1.30 – 2 pm. Mice were given an i.p. dose of insulin (Actrapid) at 2pm (1.25U/kg for males, 0.75U/kg for females). Blood glucose was measured at 15, 30,

45, 60, 90, 120, 150 and 180 minutes after insulin injection. If blood glucose dropped below 2.5mM, mice were given a glucose rescue and excluded from the study. All glucose measurements were performed with a Glucomen Areo Glucometer (A Menarini Diagnostics).

5.3.5 24 hour Feeding Studies

Mice were singly housed for 24 hours, with 6 – 11g of food provided. White absorbent pads were used instead of sawdust bedding, so crumbs of food could be collected and food intake accurately measured. Food intake was measured at the end of the 24 hour period.

5.3.6 Fast Refeed Studies

Mice were fasted at 5pm. At 8am the next day, mice were placed into individual cages, with white absorbent pads. Lids were placed on cages so animals could not smell food in the room. Animals were refeed at 9am and food intake measured after 30, 60, 120 and 180 minutes.

5.3.7 Glucose preference study

From around 6 weeks of age, mice were given 2 additional bottles of water in their cage. Water intake was measured over 48 hours to confirm there was no side preference. At around 8 weeks of age, mice were given a choice between bottles containing 8% glucose and 0.1% sucralose (and a water bottle), or 8% glucose and 8% fructose (and a water bottle), concentrations that had previously been shown to give a glucose preference of around 70% in Wild Type mice (Ogunnowo-Bada unpublished). After 24 hours, intake was measured and the sides of the bottles in the cage were switched. Intake was measured again after 24 hours. Total fluid consumption measured by bottle weight was calculated, with spillage subtraction performed (estimated using identical bottles in empty cages, handled in the same way by animal technicians). Animals were given 4 – 5 days with two water bottles as a wash out period. At around 9 weeks of age, animals were given the combination of bottles they had not received (the order of fructose or sucralose was randomly chosen).

5.3.8 Glucoprivic feeding

Animals were fasted from 8am. At 12pm, 2 – deoxy – D – glucose (2DG, 250 or 400mg/kg) or saline was injected i.p.. Mice were refeed and food intake measured at 1, 2, 3 and 4 hours. Mice were not used for further study after injection with 2DG.

5.3.9 Statistics and Data Analysis

Glucose, insulin and pyruvate tolerance tests, glucose preference studies, fast refeed studies, and glucoprivic feeding studies and body weight data were analysed using Two Way ANOVA. Food intake, 6 hour fasting blood glucose, area under the curve and % fall in blood glucose after insulin injection data were all analysed by One Way ANOVA.

5.3.10 Supervision and study assistance.

All genotyping was performed by Chris Riches. Assistance was provided by Chris Riches, Emmanuel Ogunnowo – Bada and Aureliane Pierret in some studies. Fasting in some studies was conducted by Animal Technicians within the animal facilities.

5.4 Results

5.4.1 Determining success of AgRP GK KO

When generating a new mouse line, it is important to confirm that Cre mediated recombination has occurred. I undertook two different approaches in an attempt to confirm that the AgRP GK KO was successful

5.4.1.1 In situ hybridisation

Other groups have successfully used in situ hybridisation to probe for glucokinase expression (Dunn-Meynell et al., 2002; Stanley et al., 2013). I purchased probes to detect glucokinase from Exiqon. The Exiqon probes make use of new technology, which compared to traditional approaches, means shorter nucleic acid probes can be used, with shorter incubation times. However after extensive optimisation, comparing probe binding between Wild Type and whole brain GK KO mice (previously shown to be a successful KO at the mRNA level), I was unable to detect a signal in the hypothalamus of Wild Type mice (data not shown). Glucokinase expression is known to be relatively low and so can be challenging to detect (Dunn-Meynell et al., 2002; Stanley et al., 2013).

5.4.1.2 qPCR

Using primers previously used to confirm a whole brain GK KO mouse model (Ogunnowo – Bada, unpublished), I began to measure glucokinase expression in arcuate nucleus samples taken from control and AgRP GK KO mice. I had not completed these experiments at the time of writing my thesis. However, this technique is only able to detect a reduction in GK expression in the whole arcuate nucleus, not AgRP neurons specifically, so while it may be

reassuring to see a reduction in GK expression in arcuate nuclei from AgRP GK KO mice, it does not prove that GK has been knocked out in AgRP neurons specifically.

5.4.1.3 Other options considered but not used

Another possibility would be to generate a mouse that expresses a fluorescent protein upon Cre mediated recombination. qPCR could also be performed on pools of fluorescently tagged AgRP neurons, if these cells were correctly labelled. Had I known the in situ hybridisation would have been unsuccessful, I would have explored these options. However, as they would have required different crosses, they would have had to be started at the beginning of the experiments.

Knock outs can also be detecting by testing for the absence of protein using immunofluorescence. To our knowledge as a lab, there are few, if any, reliable antibodies that exist that can differentiate between hexokinases and glucokinase. Using these antibodies may generate a false negative, as hexokinases should still be expressed in AgRP neurons of AgRP GK KO mice.

In summary, at the time of completing my thesis, I had not confirmed whether I had successfully knocked out glucokinase from AgRP neurons.

5.4.2 Body Weights in AgRP GK KO Mice

AgRP GK KO Mice appeared normal compared to controls, with no noticeable abnormalities. There were no statistically significant differences in body weight between genotypes in either gender, although AgRP – Cre Control mice appeared slightly heavier than mice of other genotypes (Figure 5-1.)

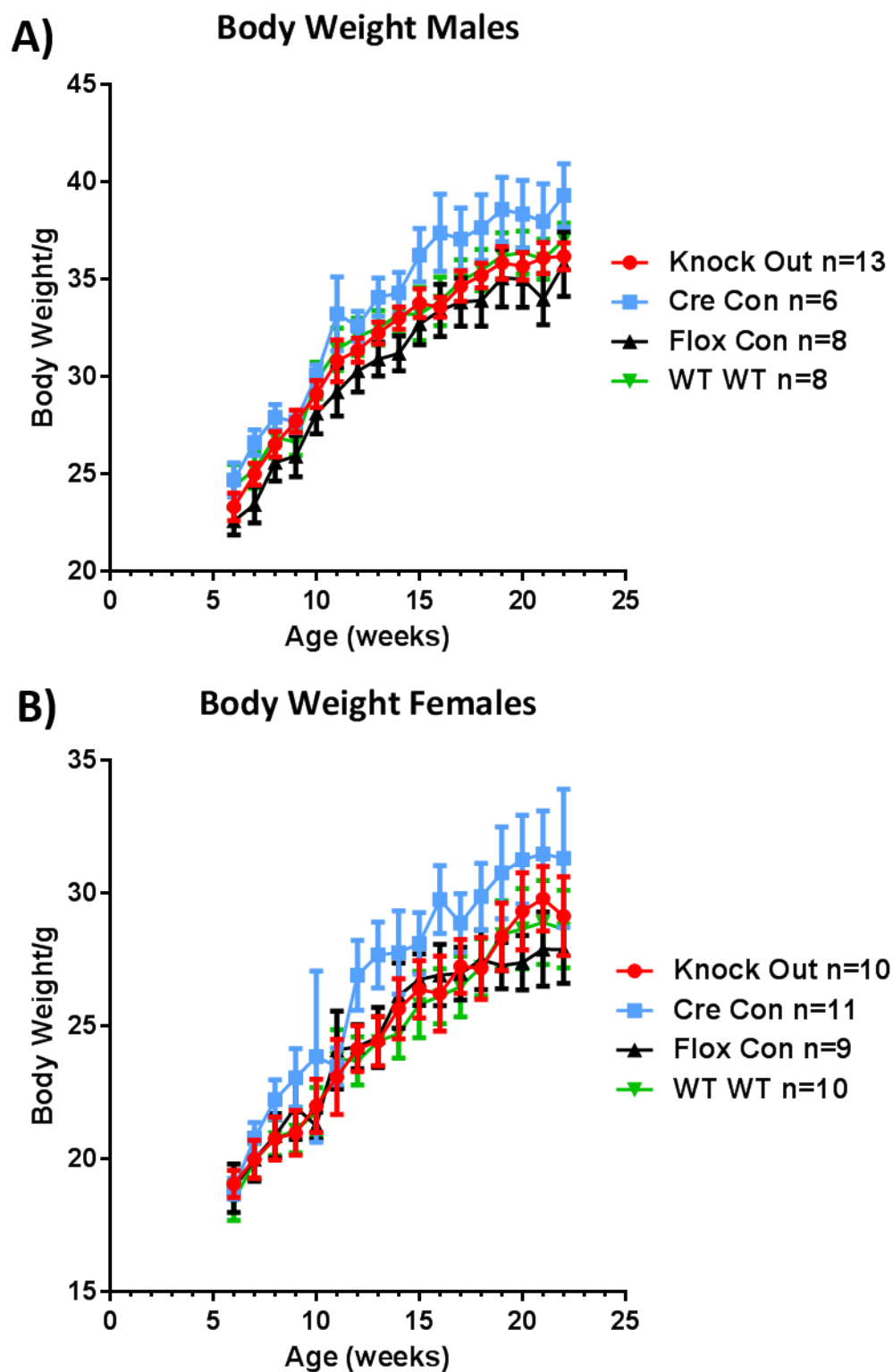


Figure 5-1: Body Weights from 6 – 23 weeks of age for AgRP GK KO mice and controls.

A) Males, and B) Females. All data points are average body weight at time point, \pm SEM.

5.4.3 High 6 hour fasting blood glucose concentrations

At the start of all studies to assess glucose tolerance, baseline blood glucose readings were taken after the 6hr fast. In initial studies on 6 – 10 week old mice (Males Figure 5-2A and Females Figure 5-3A), some of the mice had higher fasting blood glucose concentrations than expected. In the majority of mice, readings were taken on more than one occasion (up to three) and were reproducible. From previous studies in our lab, and from studies on AgRP – Cre mice in chapter 4, a typical 6 hr fasting blood glucose concentration is below 12mM glucose. The high readings are more noticeable in male mice (Figure 5-2A), with only a few females (Figure 5-3A) having baseline readings over 12mM. Data in these figures are presented as box and whisker plots, with the tails showing the maximum and minimum readings. In males, there is no statistically significant difference in baseline concentrations between genotypes, although the highest individual readings were seen in the AgRP GK KO mice (highest mouse had a reading of 28.8mM on average). In females, at 6 – 10 weeks of age, there is a statistically significant difference between the genotypes, but multiple comparison tests do not reveal differences between specific genotypes.

The same measurements were collected when performing glucose and insulin tolerance tests on 18 – 25 week old mice (Males Figure 5-2B and Females Figure 5-3B). The majority of animals had lower readings at this age. There was no statistically significant difference between genotypes in the male mice, but there was a statistically significant difference between female AgRP GK KO mice, and the AgRP Cre Control and GK Floxed Control groups. There was no statistically significant difference between AgRP GK KO mice and Wild Type control mice (Figure 5-3B). Three female mice at 18 – 25 weeks of age had particularly high blood glucose readings, two female AgRP GK KO female mice had average readings of 16 and 15mM, and one Floxed control mice, had readings of 13mM on average. When these mice are excluded, the significant difference disappears between the groups. Explanations for these high glucose readings are explored in the discussion.

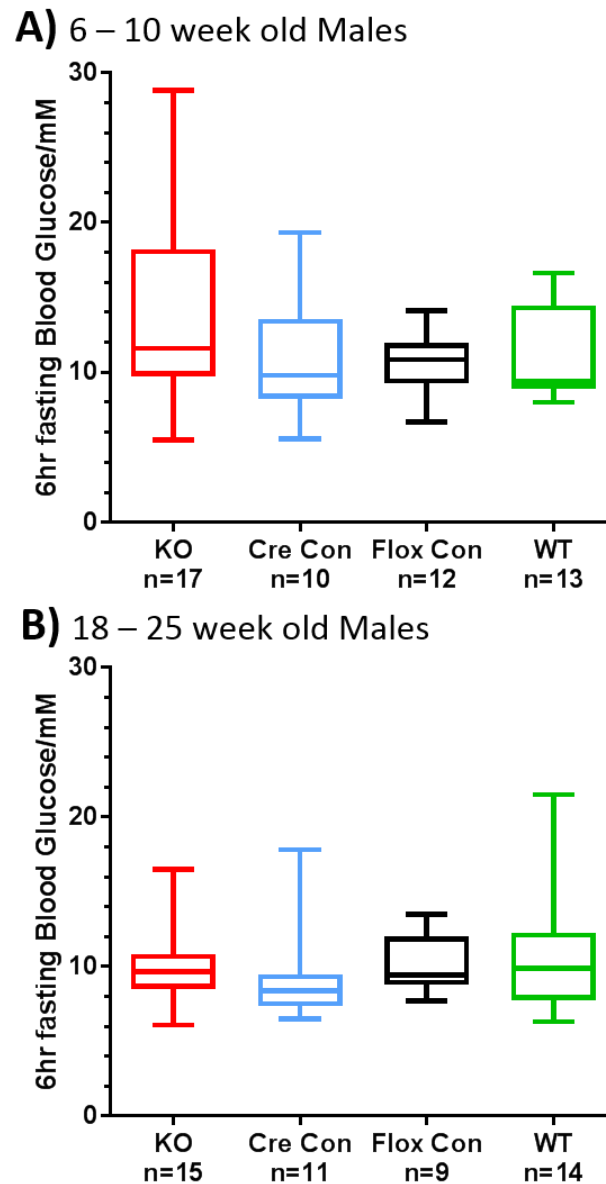


Figure 5-2: 6hr Fasting Blood Glucose in male AgRP GK KO mice and controls.

Box and whisker plots for 6hr fasting blood glucose in AgRP GK KO male mice and controls at A) 6 - 10 weeks of age and B) 18 – 25 weeks of age. Fast began at 8am. Readings were collected between 1.30 and 2pm. Data represents the average of 1 – 3 experiments for an individual animal. Data were analysed using One Way ANOVA, followed by Tukey's multiple comparisons test.

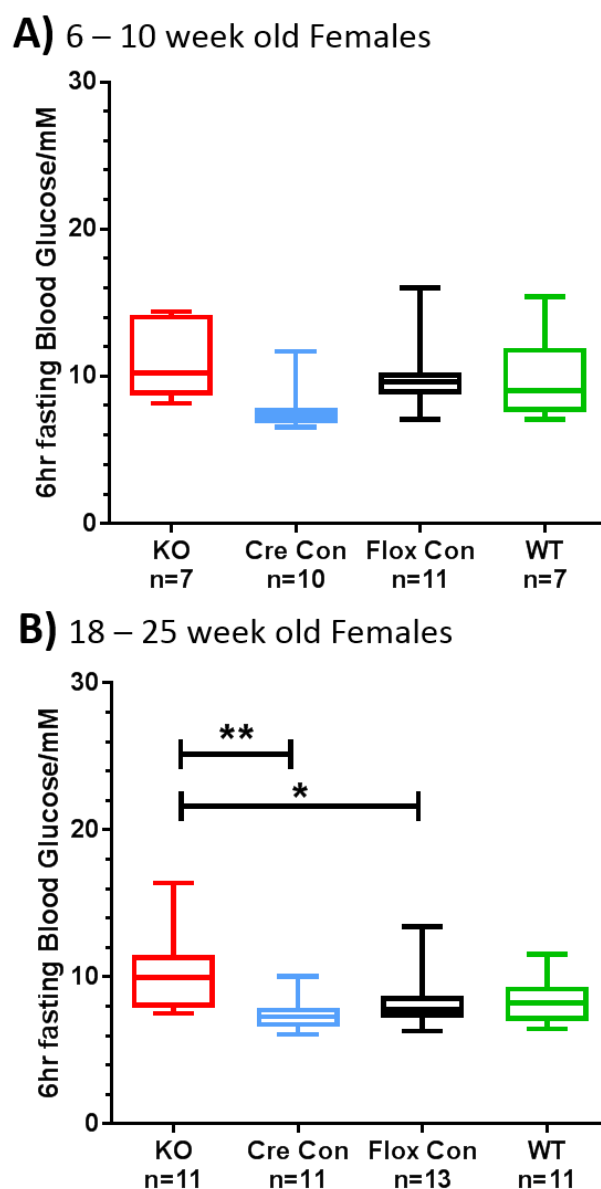


Figure 5-3: 6hr Fasting Blood Glucose in female AgRP GK KO mice and controls.

Box and whisker plots for 6hr fasting blood glucose in AgRP GK KO female mice and controls at A) 6 – 10 weeks of age and B) at 18 - 25 weeks of age. Fast began at 8am. Readings were collected between 1.30 and 2pm. Data represents the average of 1 – 3 experiments for an individual animal. Data were analysed using One Way ANOVA, followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$.

5.4.4 Normal glucose tolerance in Male AgRP GK KO Mice

Glucose tolerance was assessed in AgRP GK KO Mice and controls. In 6 – 10 week old AgRP GK KO male mice, there was no significant difference in glucose tolerance compared to control mice. This was assessed by a glucose tolerance test after a 6 hour fast (Figure 5-4A

and B), with no differences observed when comparing glucose curves using Two Way ANOVA, or comparing the area under the curve of the same data using One Way ANOVA. Three AgRP GK KO mice had a glucose reading(s) off the scale of the glucometer after glucose injection. For the purposes of the graphs, these were recorded as 33.3mM, the maximum reading on the glucometer. The AgRP GK KO mice had baseline glucose readings of 29.8, 23.8 and 19.4mM.

When phenotyping our whole brain GK KO mice, we identified differences in glucose tolerance between 6 – 10 and 18 – 25 week old mice, suggesting brain GK KO mice develop phenotypes with age. Glucose tolerance was assessed in the 18 – 25 week old AgRP GK KO mice, using glucose and insulin tolerance tests. There was no difference in glucose tolerance between genotypes in 18 – 25 week old male mice (Figure 5-4C and D).

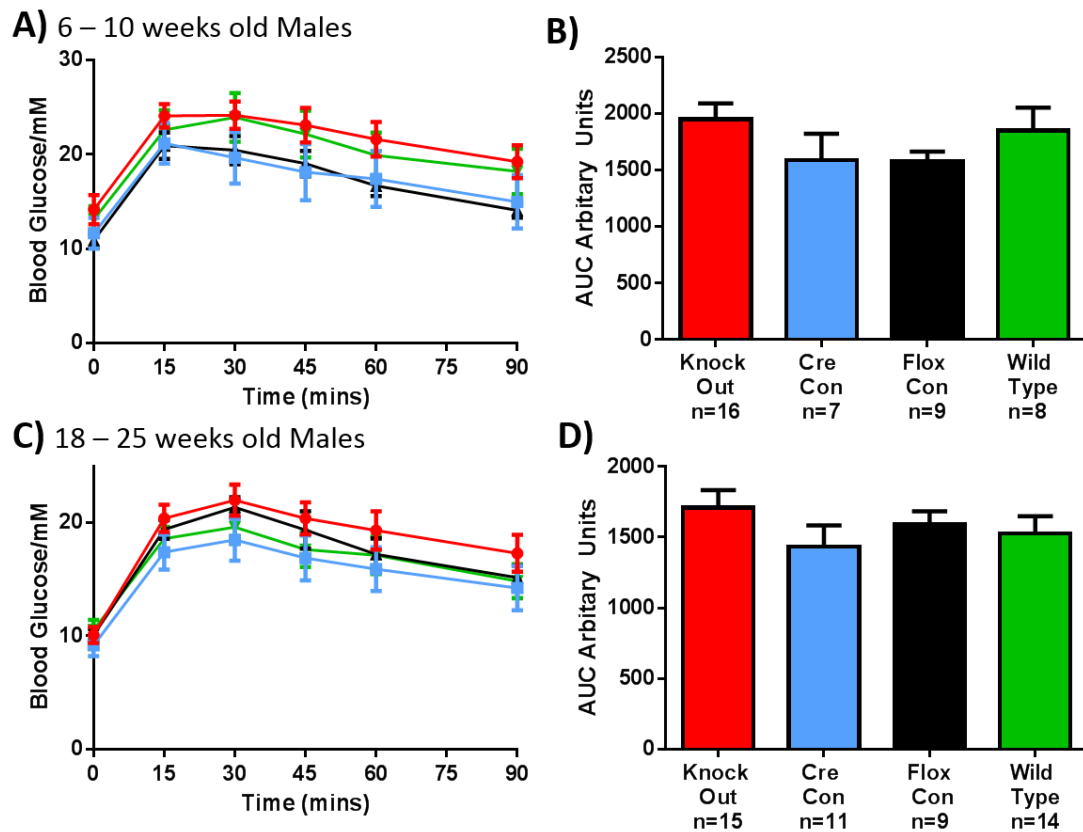


Figure 5-4: AgRP GK KO Glucose Tolerance Tests in Male Mice.

Top (A and B) 6 - 10 weeks old and Bottom (C and D) 18 – 25 week old mice. A) Blood glucose concentrations in 6 – 10 week old male mice after 1g/kg glucose i.p., and B) Area under the curve for the same data. C) Blood glucose concentrations in 18 – 25 week old male mice after 1g/kg glucose i.p., and D) Area under the curve for the same data. A and C analysed using Two Way ANOVA, B and D analysed using One Way ANOVA. All data points in A) and C) are average blood glucose concentration at time point, \pm SEM, B) and D) Mean AUC \pm SEM.

Insulin tolerance tests were used to assess insulin sensitivity (Figure 5-5) in 6 – 10 and 18 – 25 week old male mice. There was no significant difference in insulin tolerance between AgRP GK KO and control mice at either age when assessed by Two Way ANOVA. Given that the baseline glucose concentrations are variable between mice, it is hard to determine from this analysis the effect insulin is having. Comparing the percentage fall in blood glucose concentrations can take into account the variable baselines. The nadir of all genotypes in male mice appears to be at 60 minutes at both ages. There was no difference in the percentage fall in blood glucose concentrations after 45 or 60 minutes in male mice, when compared using One Way ANOVA (data not shown), at either age. Data from mice that were

rescued due to low blood glucose concentrations were not included in all the analyses, nor were mice with a negative insulin sensitivity (i.e.: blood glucose concentrations rose).

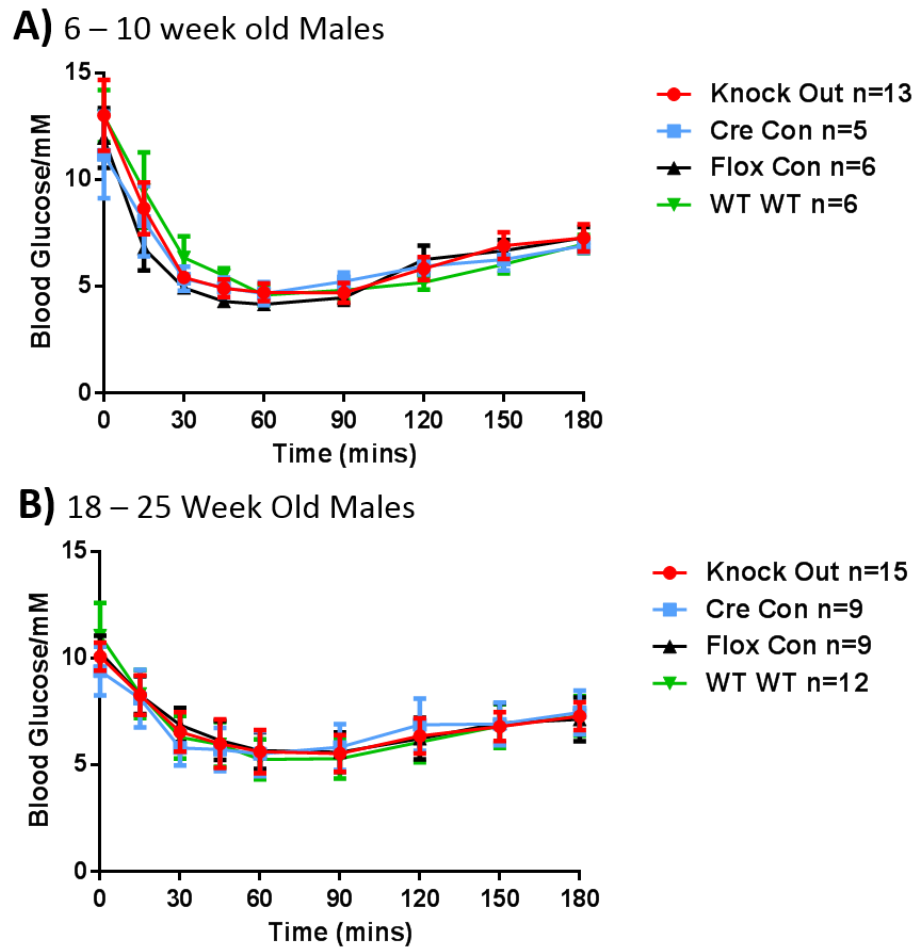


Figure 5-5: AgRP GK KO Insulin Tolerance Tests in Male Mice.

A) Blood glucose concentrations in male (6 - 10 weeks) mice after 1.25U/kg insulin i.p., B) Blood glucose concentrations in male (18 – 25 weeks) mice after 1.25U/kg insulin i.p., A and B analysed using Two Way ANOVA, A) and B) are average blood glucose concentration at time point, \pm SEM.

AgRP neurons have been shown to be involved in controlling HGP (Könner et al., 2007). A crude assessment of hepatic glucose production is a pyruvate tolerance test. A pyruvate bolus is given, that is metabolised by the liver to form glucose. A higher AUC would suggest that an animal has increased capacity for HGP. There was no difference in HGP between genotypes, in male mice (Figure 5-6). The baseline glucose readings for AgRP GK KO mice are higher in this study, which may cause the higher AUC. When corrected for the different baseline, to test for the absolute glucose increase upon pyruvate injection, there was no difference in AUC between genotypes (data not shown).

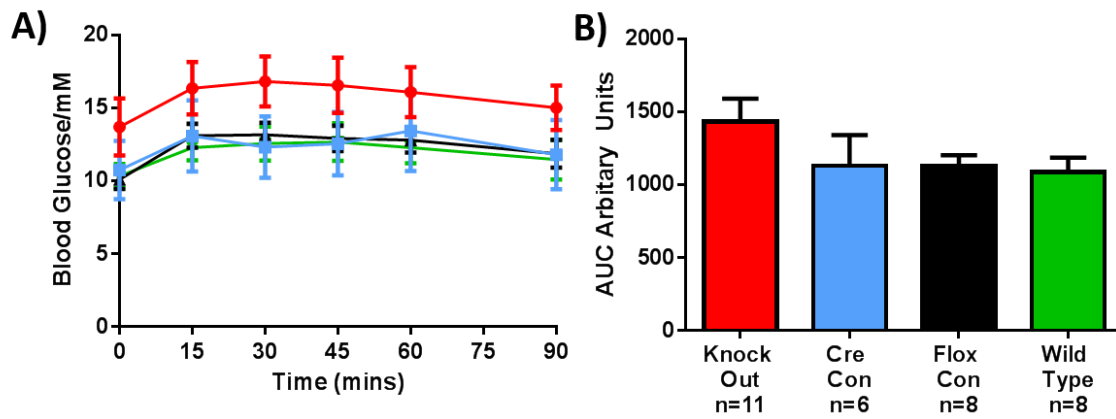


Figure 5-6: AgRP GK KO Pyruvate Tolerance Tests in 6 – 10 week old Male Mice.

A) Blood glucose concentrations in 6 - 10 week old male mice after 1g/kg pyruvate i.p., and B) Area under the curve for the same data. A) analysed using Two Way ANOVA, B) analysed using One Way ANOVA. All data points in A) are average blood glucose concentration at time point, \pm SEM, B) Mean AUC \pm SEM.

5.4.5 Assessing glucose tolerance in female AgRP GK KO mice

In 6 – 10 week female mice, there was no difference in glucose tolerance between genotypes as assessed by a glucose tolerance test after a 6 hour fast (Figure 5-7A and B) but 18 – 25 week old female AgRP GK KO mice were mildly glucose intolerant (Figure 5-7C and D) compared to controls. Two Way ANOVA with Tukey post hoc comparison revealed a significant difference between the knock outs and all other genotypes at 45 and 60 minutes. When assessed using AUC, using One Way ANOVA with Tukey test for multiple comparisons, there was a significant difference between the Knock out and Cre control group.

Given that there was a statistically significant difference in baseline blood glucose concentrations in 18 – 25 week old female mice with a 6hr fast (Figure 5-3B) I normalised the baselines to look at absolute changes in blood glucose concentrations (data not shown). The overall difference as assessed by Two Way ANOVA between groups disappeared. The difference as assessed by One Way ANOVA in AUC disappeared (data not shown).

Of the two female AgRP GK KO mice that had elevated fasting blood glucose concentrations (discussed earlier), one had glucose readings off the scale of the glucometer for some of the study (for the purposes of the graphs it was recorded as 33.3mM, the maximum reading on the glucometer). This AgRP GK KO mice had a baseline glucose reading of 17mM in this

study. This mouse may be an outlier. It is difficult to exclude these mice without further tests, and without confirming the success of the AgRP GK KO. When the two mice with higher fasting glucose concentrations are excluded, the GTT result is not statistically significant.

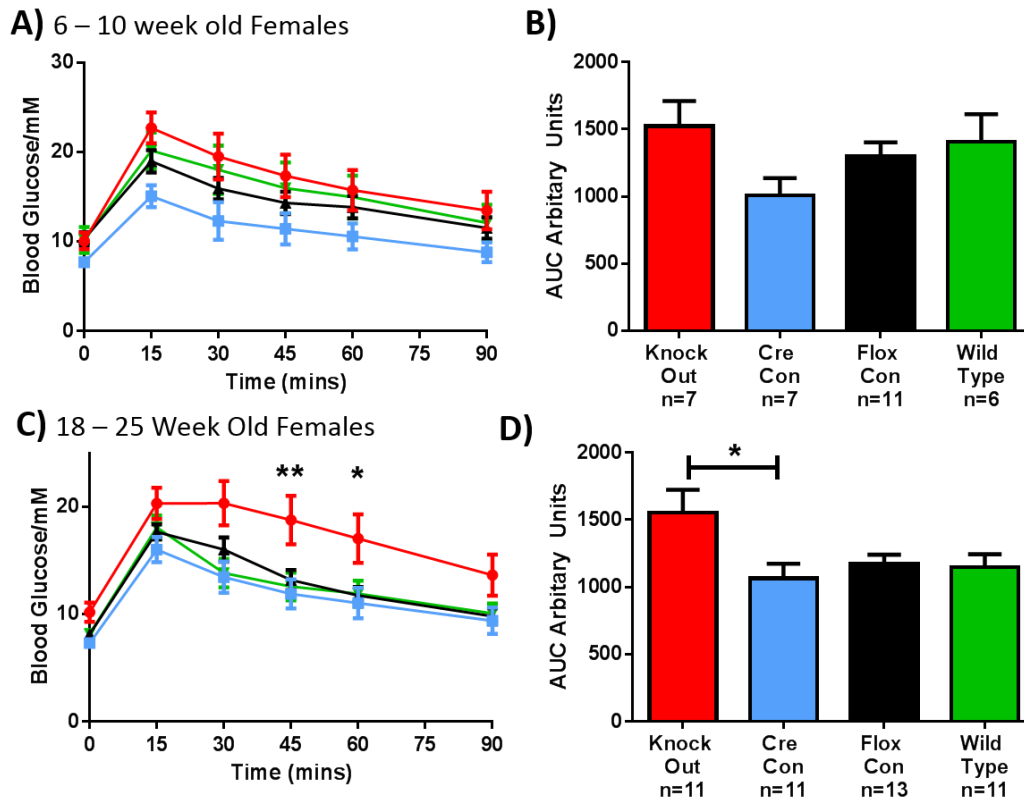


Figure 5-7: AgRP GK KO glucose tolerance tests in Female mice

A) Blood glucose concentrations in female (6 – 10 week old) mice after 1g/kg glucose i.p., and B) Area under the curve for the same data. C) Blood glucose concentrations in female (18 – 25 week old) mice after 1g/kg glucose i.p., and D) Area under the curve for the same data. A and C analysed using Two Way ANOVA with Tukey Test for multiple comparisons, B and D analysed using One Way ANOVA with Tukey Test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$. All data points in A) and C) are average blood glucose concentration at time point, \pm SEM, B) and D) Mean AUC \pm SEM.

There was no significant difference in insulin tolerance in female mice, at 6 – 10 or 18 – 25 weeks of age, when assessed by Two Way ANOVA (Figure 5-8A and C). As for males, the percentage fall in blood glucose concentrations was compared (Figure 5-8B and D). The nadir of three genotypes at 6 – 10 weeks appears to be at 30 minutes. There is a statistically

significant difference between the genotypes at 6 – 10 weeks, as assessed by One Way ANOVA. Tukey's multiple comparison test reports a significant difference between the Knock out and Floxed control group. At 45 minutes, in addition to this difference, there is a significant difference between the Cre Control and Knock Out groups. The data for 30 minutes are presented in the graph (Figure 5-8B). This suggests AgRP GK KO female mice are more sensitive to insulin than controls, between 6 – 10 weeks of age. Data from mice that were rescued due to low blood glucose concentrations were not included in all the analyses, nor were mice with a negative insulin sensitivity (i.e.: blood glucose concentrations rose). n values are low for all genotypes except the Floxed controls, meaning some of the assumptions made for use of a parametric statistical test to be valid are not met. If a Kruskal Wallis Test is used, a non parametric test, there is still a statistically significant difference between the genotypes in 6 – 10 week old female mice at 30 and 45 minutes. A Dunn's multiple comparison test reports significant differences between the Knock Out, and the Cre and Floxed Controls at 30 minutes, but does not report any differences between the genotypes at 45 minutes. However, as I do expect the data to be normally distributed, it would be more appropriate to use a parametric statistical test. Testing more animals will be necessary before a conclusion can be drawn for this study.

There was no difference in the percentage fall in blood glucose at 45 or 60 minutes (Figure 5-8D) in 18 – 25 week old female mice. The nadirs for the different groups in the female mice are variable, making a reliable analysis slightly more challenging. However, the floxed control and knock out groups, which have the largest percentage fall, both reached their group average nadir at 45 minutes, and there is no difference between these groups at this time point. Some negative values were excluded from the percentage fall in blood glucose plot, but were included in the statistical analysis.

In summary, the data suggests that 18 – 25 week old AgRP GK KO female mice may be glucose intolerant, a conclusion reliant on the success of the knock out being confirmed. 6 – 10 week old AgRP GK KO female mice may have increased insulin sensitivity. More mice must be studied to confirm this finding.

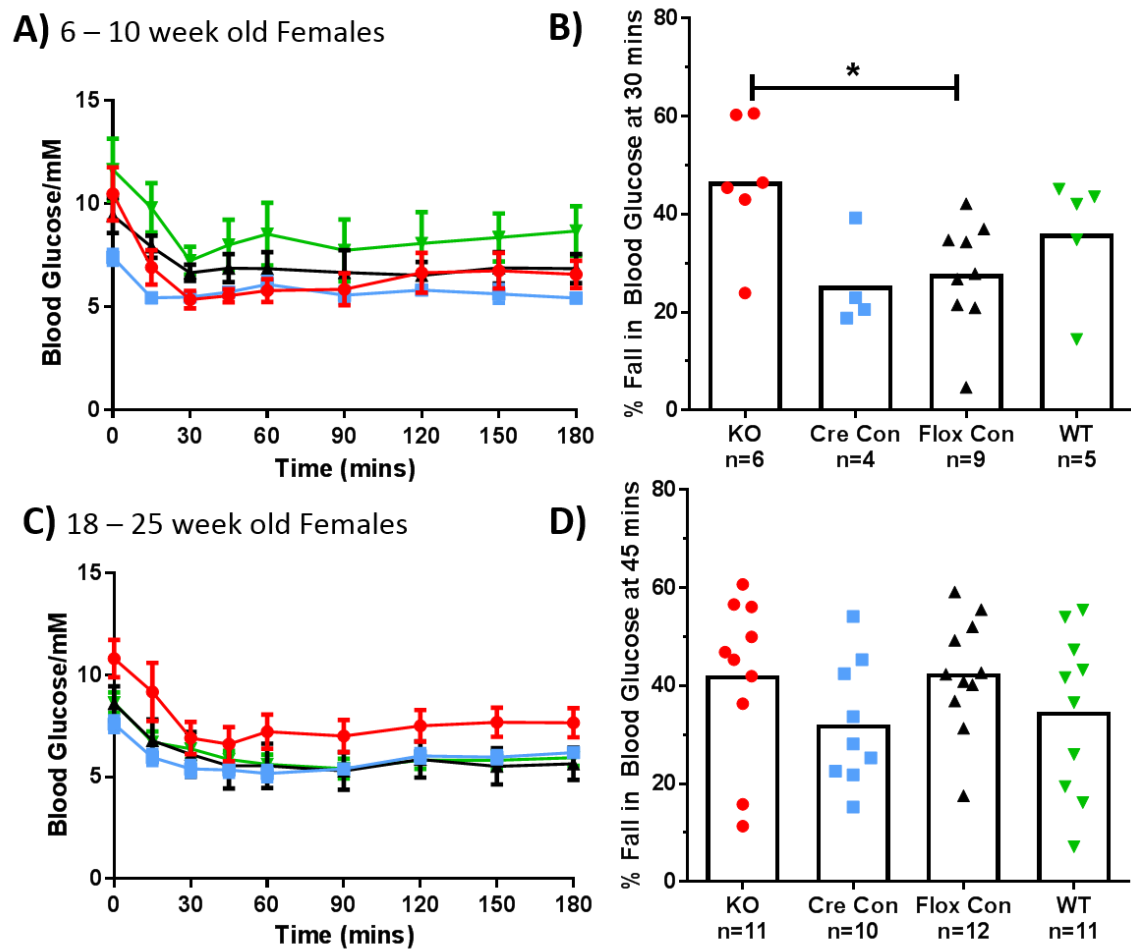


Figure 5-8: AgRP GK KO Insulin Tolerance Tests in Female Mice

A) Blood glucose concentrations in female (6 – 10 week old) mice after 0.75U/kg insulin, i.p., B) Percentage fall in blood glucose concentration 30 minutes after insulin injection compared to baseline, in female (6 – 10 week old) mice. C) Blood glucose concentrations in female (18 – 25 week old) mice after 0.75U/kg insulin, i.p., and D) Percentage fall in blood glucose concentration 45 minutes after insulin injection compared to baseline, in female (18 – 25 week old) mice. A and C analysed using Two Way ANOVA, B and D analysed using One Way ANOVA with Tukeys multiple comparisons test. All data points in A) and C) are average blood glucose concentration at time point, \pm SEM. Data points in B) and D) represent % fall in blood glucose of individual animals, bars represent average for genotype. * $p < 0.05$

There was no difference in pyruvate tolerance between genotypes in female mice tested at 6 – 10 weeks of age (Figure 5-9). When corrected for the different baseline glucose readings, there was still no difference between genotypes in AUC.

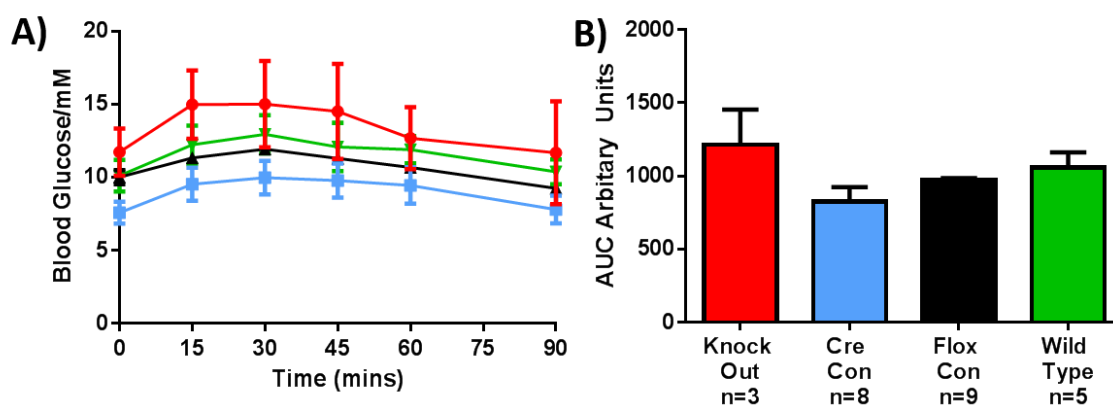


Figure 5-9: AgRP GK KO Pyruvate Tolerance Tests in 6 – 10 week old Female Mice.

A) Blood glucose concentrations in (6 - 10 week old) female mice after 1g/kg pyruvate i.p., and B) Area under the curve for the same data. A) analysed using Two Way ANOVA, B) analysed using One Way ANOVA. All data points in A) are average blood glucose concentration at time point, \pm SEM, B) Mean AUC \pm SEM.

5.4.6 No differences in feeding behaviour in AgRP GK KO Mice

Feeding behaviour in AgRP GK KO Mice was then assessed. Food intake over 24 hours was measured. Mice were singly housed in cages with white pads. These pads allow crumbs to be collected so food intake can be accurately measured. However, splitting animals from their usual group housing and placing them in a new environment may lead to stress, meaning food intake measurements may not reflect animal's usual food intake. There was no difference in food intake between genotypes in male mice, at either age tested (Figure 5-10A and C), or female mice, at either age tested (Figure 5-11A and C).

AgRP neurons become more active in the fasting state due to hormonal factors such as ghrelin increasing in concentration (Takahashi and Cone, 2005; Yang et al., 2011). Glucose inhibition of these neurons may also play a role (Hao et al., 2016; Murphy et al., 2009a). Without glucokinase, AgRP neurons may perceive glucose concentrations to be lower than they actually are, which may cause a larger refeed after a fast. However, there was no difference in the refeed after a fast between genotypes in male mice, at either age tested (Figure 5-10B and D), or female mice, at either age tested (Figure 5-11B and D).

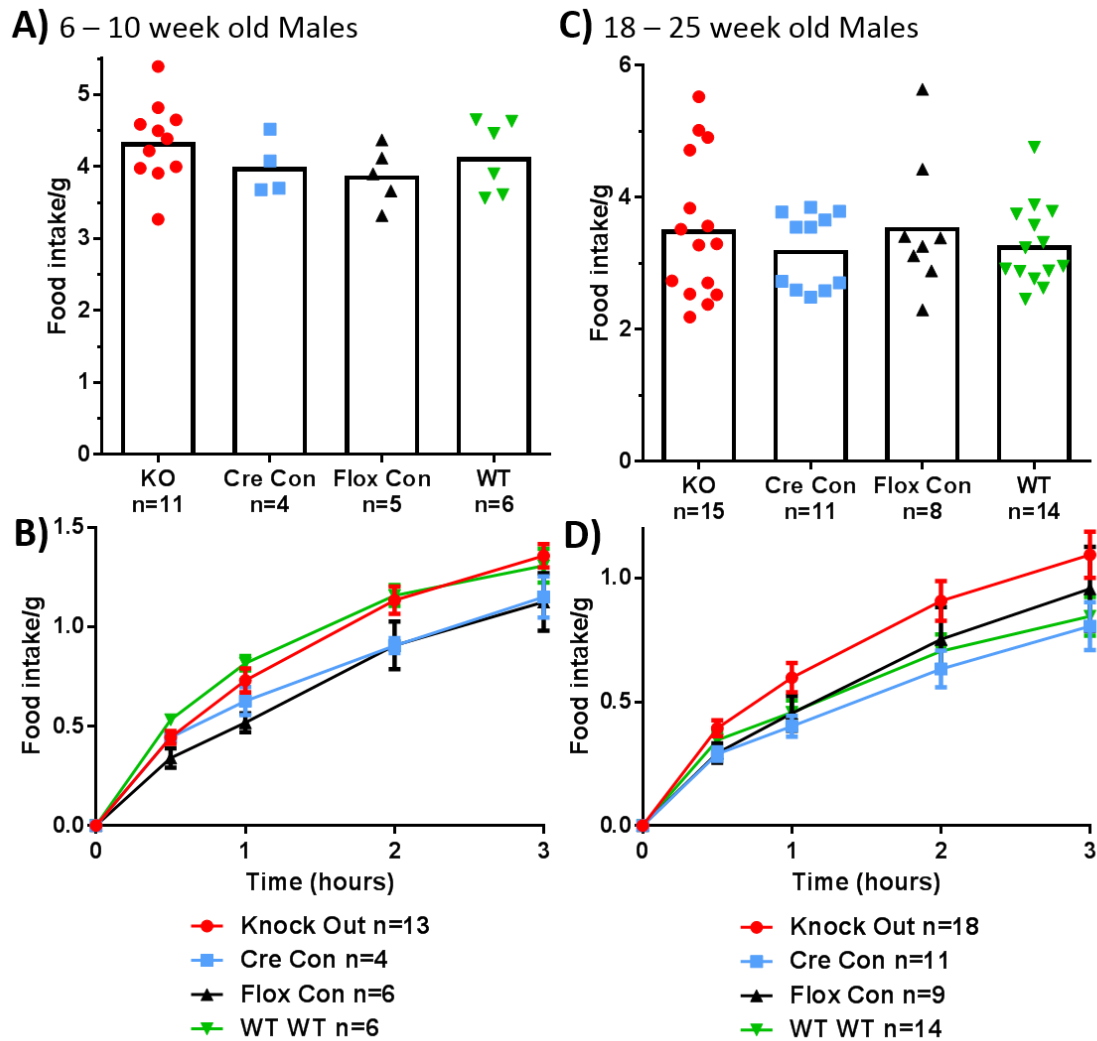


Figure 5-10: Feeding Studies in Male AgRP GK KO Mice

A) Food intake in Male (6 – 10 week old) mice measured over 24 hours, B) Food intake measured over 3 hours after an overnight fast in male (6 – 10 week old) mice, C) Food intake in Male (18 – 25 week old) mice measured over 24 hours, D) Food intake measured over 3 hours after an overnight fast in male (18 – 25 week old) mice. A and C Analysed using One Way ANOVA. Data points in A and C represent food intake of individual animals. Bars represent average for genotype, \pm SEM. B and D Analysed using Two Way ANOVA. All data points in B and D are average food intake at time point.

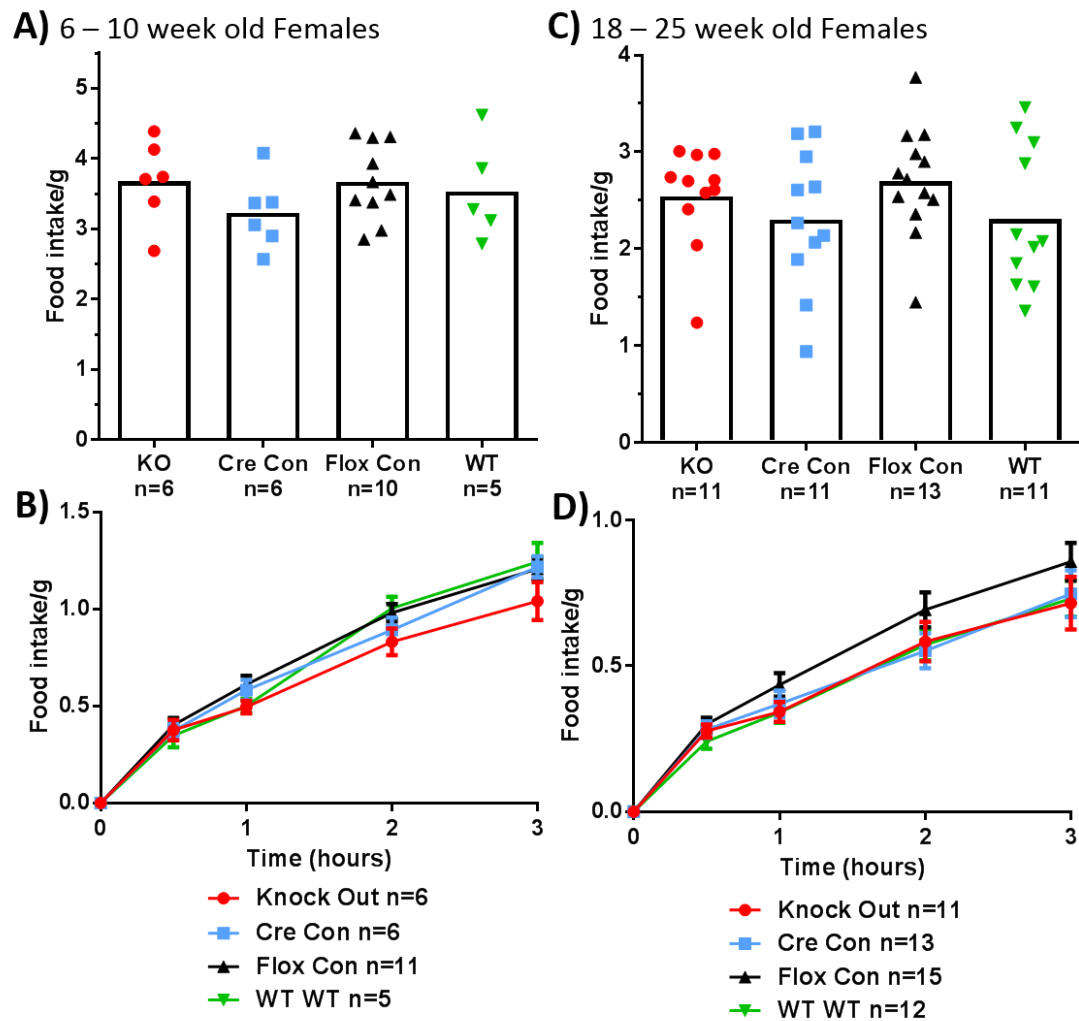


Figure 5-11: Feeding Studies in Female AgRP GK KO Mice.

A) Food intake in Female (6 – 10 week old) mice measured over 24 hours, B) Food intake measured over 3 hours after an overnight fast in female (6 – 10 week old) mice, C) Food intake in Female (18 – 25 week old) mice measured over 24 hours. D) Food intake measured over 3 hours after an overnight fast in female (18 – 25 week old) mice. A and C Analysed using One Way ANOVA. Data points in A and C represent food intake of individual animals, bars represent average for genotype, \pm SEM. B and D Analysed using Two Way ANOVA. All data points in B and D are average food intake at time point.

5.4.7 No difference in glucose preference in AgRP GK KO Mice

Increased consumption of sugary drinks has contributed to the obesity epidemic (Malik et al., 2006). Understanding what drives increased sugar consumption may aid the development of therapies to tackle obesity. Brain GK KO mice have an increased glucose preference compared to control genotypes, when they can choose between glucose and

either fructose, or the artificial sweetener sucralose. However, there was no statistically significant difference in glucose preference between AgRP GK KO mice and controls in either preference assay (Figure 5-12).

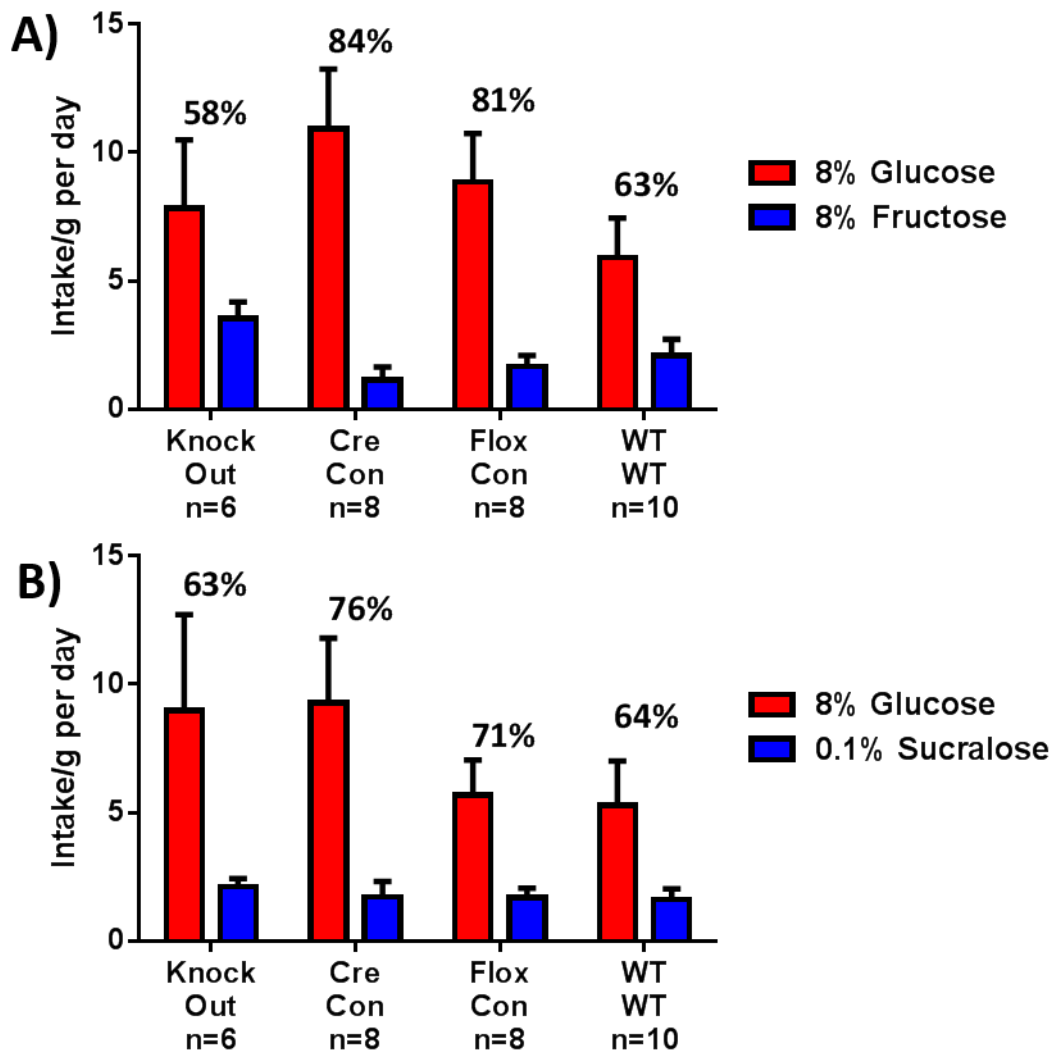


Figure 5-12: Glucose Preference Studies in Male Mice (6 - 10 weeks old).

A) Comparing intake of 8% Glucose and 8% Fructose, B) Comparing intake of 8% Glucose and 0.1% Sucralose. Bars represent mean fluid intake \pm SEM. Values above bars represent preference for glucose over other drink. Analysed by Two Way ANOVA.

5.4.8 Unable to assess differences in glucoprivic feeding in AgRP GK KO Mice

Low brain glucose levels cause a feeding response (Dunn-Meynell et al., 2009), in an attempt to restore brain glucose concentrations through food consumption. This can be mimicked by injection of the non metabolisable glucose analogue 2DG, into the brain or intraperitoneally (Borg et al., 1995; Miselis and Epstein, 1975; Stanley et al., 2016). Using a mix of genotypes,

two doses of 2DG i.p., were tested, with hourly monitoring of food intake for four hours post injection. A dose of 250mg/kg or 400mg/kg did not elicit a feeding response that was statistically larger than saline control injections (Figure 5-13). However, there was a trend towards increased feeding with 2DG injection at the final time point in female mice with a dose of 250mg/kg (Figure 5-13B). As no increase in feeding with 2DG treatment was observed, it was judged that the experimental design could not be used to test the effect of AgRP GK KO on glucoprivic feeding.

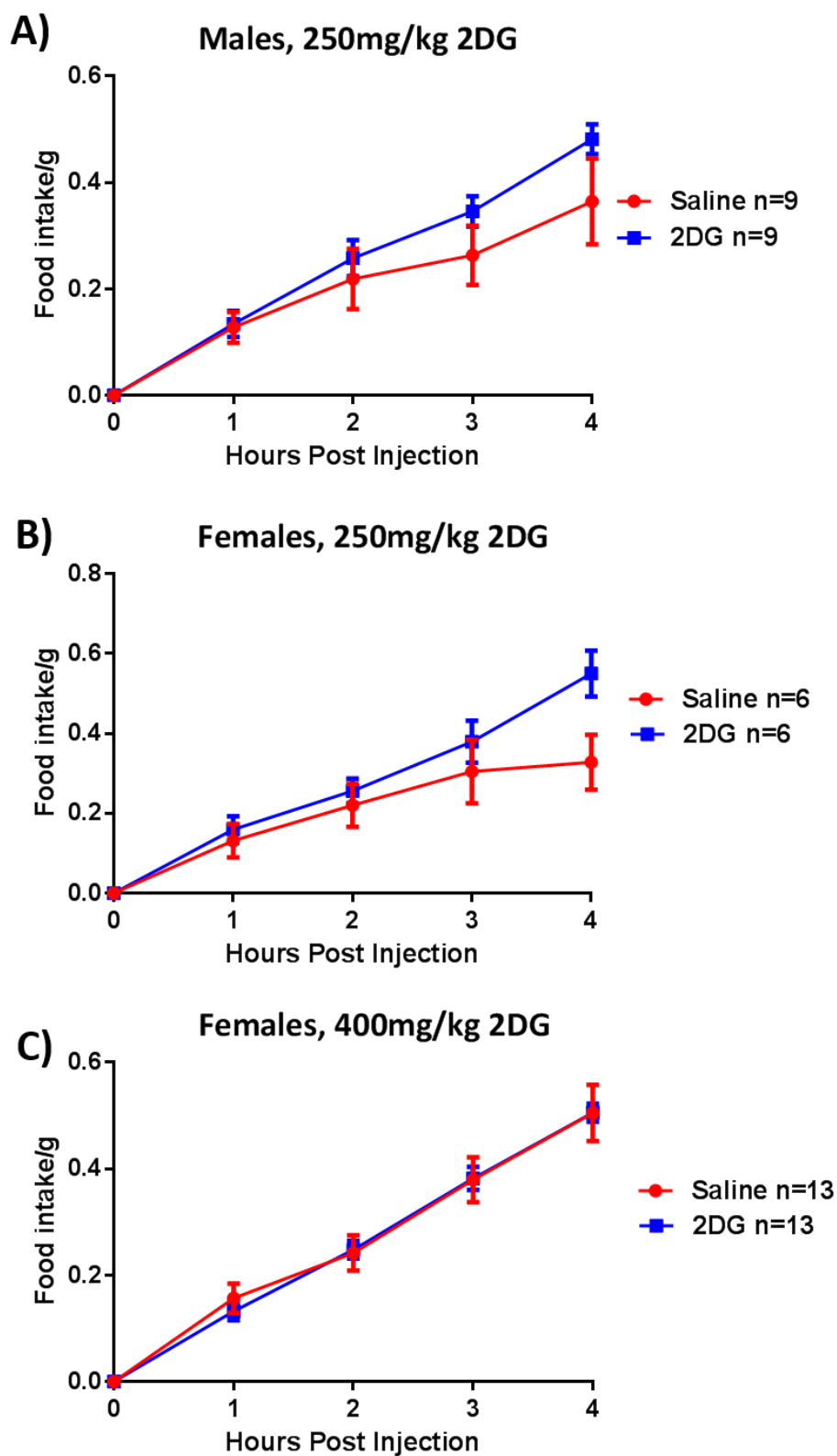


Figure 5-13: Glucoprivic Feeding Studies in 6 - 10 week old Mice.

A) Males treated with 250mg/kg 2DG or Saline, i.p., B) Females treated with 250mg/kg 2DG or Saline i.p., C) Females treated with 400mg/kg 2DG or Saline i.p. Food intake compared using Two Way ANOVA. All data points are average food intake at time point, \pm SEM

5.5 Discussion

The preliminary results presented in this chapter suggest that glucokinase expressed in AgRP neurons may be important in the regulation of glucose homeostasis. However, these results cannot be concluded until further studies to increase n values in 6 – 10 week old female mice take place, and until the success of the glucokinase knock out from AgRP neurons has been confirmed.

If these preliminary results are confirmed, further studies, such as euglycaemic hyperinsulinemic or hyperglycaemic clamps could be used to identify what specific aspect of glucose handling has been altered by the AgRP GK KO. AgRP neurons have been shown to act to control blood glucose homeostasis at three principal levels: controlling HGP, controlling BAT insulin sensitivity and controlling skeletal muscle insulin sensitivity, as discussed in chapter 4. The data here suggest glucose sensing via glucokinase by AgRP neurons does not lead to changes in HGP, as there were no differences in pyruvate tolerance in either gender, at 6 – 10 weeks of age (Figure 5-6 and Figure 5-9), although further studies are required in knock out female mice to confirm this finding. No further conclusions can be made based on this preliminary data.

If these preliminary findings are confirmed, they would show that glucokinase in AgRP neurons controls glucose homeostasis, but not other functions known to be controlled by AgRP neurons, principally feeding behaviour.

5.5.1 High baseline glucose concentrations in some mice

One confounding effect in the studies presented is the elevated fasting glucose concentrations seen in some mice, spread across the various genotypes. The effect is less pronounced in females than in males. In male mice the effect improves with age.

5.5.1.1 Differences due to successful knock out of GK

In 6 – 10 week old male mice, the highest blood glucose readings were seen in AgRP GK KO mice. Female AgRP GK KO mice have a significantly higher fasting blood glucose concentration compared to Floxed and Cre controls, but not wild type mice, at 18 – 25 weeks of age. It is possible that these AgRP GK KO mice have the most successful knock out, and that the high fasting blood glucose concentrations are a phenotype. At present, as I was unsuccessful in confirming the success of the AgRP GK KO, I cannot comment further on this possibility. However, high fasting blood glucose concentrations, although highest in the AgRP

GK KO mice, were also seen in other mice. At 18 – 25 weeks of age, in male mice, the highest average fasting blood glucose concentration was seen in a wild Type male mouse, not an AgRP GK KO mouse, suggesting another factor unrelated to the presence of glucokinase in AgRP neurons, may be causing this effect. Possible explanations for this are explored in the next section.

5.5.1.2 Differences due to genetic background of mice

To generate the AgRP GK KO mice, Glucokinase floxed mice, on a pure C57/BL6 background (Postic et al., 1999) were crossed with AgRP – Cre mice which were generated, and maintained, on a mixed background (largest percentage is C57/BL6). Different mouse strains have been reported to have differing fasting blood glucose concentrations (Andrikopoulos et al., 2005; Champy et al., 2008; La Fleur et al., 2013; Goren et al., 2004; Lin et al., 2005).

Crossing strains together can generate a spectrum of blood glucose concentrations depending on the cross, and dietary conditions (Cheverud et al., 2014; Kido et al., 2000). Random effects due to normal Mendelian genetics could have caused random combinations of alleles that cumulatively caused high blood glucose concentrations. Back crossing the mice onto a pure C57/BL6 may have solved this problem, but may not have been feasible given the short length of my PhD.

While this specific effect, of extremely high blood glucose concentrations in a few progeny from some crosses has not been reported in the literature, as can be seen from the graphs (Figure 5-4A and C), there is a minimal effect on the group average when a few mice with high starting glucose concentrations are included in graphs and statistical analyses.

It has been reported that ectopic AgRP expression and subsequent Cre mediated recombination during development can lead to unwanted phenotypes (He et al., 2016; Kuperman et al., 2016). If glucokinase was deleted from the pancreas, for instance, during development, it could lead to raised blood glucose concentrations, as has been reported (Postic et al., 1999). However, I saw raised blood glucose concentrations in Wild Type mice that do not express Cre, making this explanation unlikely. The phenotype would also be expected to worsen with age, not improve (although not all mice were measured at both ages so a good comparison of age related changes is difficult). A reduction in stress could also explain the improvement in fasting glucose in males as mice get used to being handled

over time, although not all mice studied at 18 – 25 week old were studied at 6 – 10 weeks old. In female mice though, the effect on fasting blood glucose is smaller.

The baseline glucose graphs also highlight another important point. For the female AgRP Cre mice, nearly all the blood glucose readings cluster tightly, compared to the other genotypes. Many studies using the AgRP Cre line crossed to a floxed mouse only use one control group, when there are three control groups that should be used. This does make studies more time consuming: the cross necessary to generate the 4 genotypes (as outlined in section 5.3.1) means only half the offspring can be used for studies. Our breeding strategy means studies were not performed on the same day for all mice meaning an additional level of variation is introduced. It also means that the number of mice to generate sufficient statistical power must be increased to see statistically significant results, as having three control groups compared to one increases the number of comparisons that must be done. However, as championed in a review discussing unexpected effects of Cre expression alone (Harno et al., 2013), it does allow differences that are not genuine to be identified, that would not be seen if only one control genotype was used.

5.5.2 AgRP GK KO may cause gender and age specific effects on glucose tolerance

While no phenotypes were observed in male AgRP GK KO mice, there were differences in some tests carried out in female AgRP GK KO mice. However, these results require careful interpretation, as discussed in the next sub sections.

5.5.2.1 Differences in glucose tolerance in 18 – 25 week old AgRP GK KO female mice

The difference in glucose tolerance in 18 – 25 week old AgRP GK KO female mice was only observed when two female mice that started with high blood glucose readings were included in the data set. When these mice were excluded, or the baselines of the animals were normalised, the differences in glucose tolerance disappeared. It is possible, as discussed in the previous section, that these two females have a particularly successful knock out of glucokinase. On the other hand, other confounding effects, discussed in section 5.5.3.3, may account for this difference.

The lab plans to perform additional GTT's on 18 – 25 week old female AgRP GK KO mice to see if other mice display the glucose intolerance phenotype. Confirmation of the success of the knock out in these mice will also be necessary.

5.5.2.2 Differences in insulin sensitivity in 6 – 10 week old AgRP GK KO female mice

Analysis of data from insulin tolerance tests suggested that 6 – 10 week old female AgRP GK KO mice had an increased insulin sensitivity compared to control genotypes. However, there was no difference when the data set was considered overall using Two Way ANOVA. This change in insulin sensitivity had no effect on glucose tolerance as assessed by a glucose tolerance test. To confirm the finding of increased insulin sensitivity, further mice must be tested, as the n values are too low for use of a parametric statistical test to be valid. This is the required statistical test to be used, as I expect the data would be normally distributed. Further studies are being planned by the lab to increase these n values, allowing conclusions to be drawn.

5.5.2.3 Reasons for gender differences in glucose tolerance

Phenotypes were only observed in female AgRP GK KO mice. Similar gender specific effects of a GK KO have recently been reported (Steinbusch et al., 2016). In this study, SF1 neuron GK KO altered body composition and caused impaired hypoglycaemia - induced glucagon secretion, but only in female mice. As discussed earlier, the neurons retained the ability to sense glucose, suggesting GK may play other roles in these cells, and that they employ a different glucose sensing mechanism. It cannot be said if this is the case in this AgRP GK KO mice.

Care must be taken when studying glucose tolerance in different genders (Mauvais-Jarvis et al., 2017). Differences in glucose tolerance have recently been reported by the Routh lab, demonstrating differences in VMH neuron glucose sensing, and responses to IIH (insulin induced hypoglycaemia), when female mice are at different phases of the oestrous cycle (Santiago et al., 2016a, 2016b). In their study (Steinbusch et al., 2016), and in my experiments, no control was made for the phase of the oestrous cycle that female mice were in. Additionally, in some studies, mice of both genders were studied on the same day. Although they were given up to 6 hours to acclimatise to the smells of the mice of the other gender, this may have been the first time they were exposed to the smells of mice of the other gender, due to the individually ventilated cages that mice are housed in. Again, this could lead to false positives being generated. To overcome these problems, studies could be performed at a selected phase of the oestrous cycle.

5.5.3 AgRP GK KO does not affect feeding behaviour

In both the 24 hour feeding studies and the fast refeed studies, AgRP GK KO did not affect the food intake. This might be considered surprising given the important role that AgRP neurons play in the control of food seeking behaviour (Luquet et al., 2005). However, other manipulations of AgRP neuron glucose sensing have not lead to changes in feeding behaviour (Claret et al., 2007; Smith et al., 2015). Additionally, changes in blood glucose concentration have not been reported to be an important regulator of feeding behaviour, beyond glucoprivic feeding (Dunn-Meynell et al., 2009). This is a controversial area though, as discussed in chapter 1.

In my experience with whole brain GK KO mice, an overnight fast does not cause most mice to become hypoglycaemic, and so the fast refeed paradigm may not be sufficient to assess whether low blood glucose concentrations cause mice to eat more or less in the absence of GK expression. It instead appears to show that feeding, in response to fasting related cues such as ghrelin, is normal in AgRP GK KO mice.

5.5.4 No further understanding of the role of AgRP Neurons in the control of glucoprivic feeding

Given that AgRP neurons are glucose inhibited and shown to be necessary for food intake (Luquet et al., 2005), I set out to test if the AgRP GK KO had altered glucoprivic feeding. A previous study has suggested that AgRP neurons are not necessary for glucoprivic feeding, although this was performed in a model where AgRP neurons were ablated shortly after birth, and so circuit re wiring to permit glucoprivic feeding may have taken place (Luquet et al., 2007).

There was no difference in food consumption between saline and 2DG injected mice, suggesting in our experimental set up, 2DG does not drive observable changes in feeding. The total consumed for the saline treated mice at 4 hours was larger than that seen in the previous study examining glucoprivic feeding regulation by AgRP neurons, with the same 4 hr time point (Luquet et al., 2007). This suggests the experimental design I used is causing baseline food intake to increase, meaning differences in feeding caused by 2DG cannot be observed. Disturbing the animals hourly to measure food intake may increase arousal, leading to feeding when mice otherwise would not have eaten. The lab plans to repeat these studies, with injection of 2DG followed by a single measurement of food intake after 4 hours.

5.5.5 AgRP GK KO has no effect on glucose preference

Whole brain GK KO mice have an increased preference for glucose, over sucralose or fructose. Our working hypothesis is that absence of GK causes mice to perceive that glucose concentrations are lower than they actually are, driving additional glucose intake. There is no evidence at present to suggest that this is linked to an added reward value with GK KO, or to a taste preference difference in the whole brain GK KO mice. Given that AgRP neurons drive food seeking behaviour, an inability to sense glucose in these neurons with GK KO may drive mice to drink more glucose, compared to sucralose, an artificial sweetener which does not provide energy for animals, or fructose, which has a lower post - oral reward effect (Matsumura et al., 2010; Sclafani and Ackroff, 2012; Zukerman et al., 2013).

Manipulations of arcuate GK expression have been implicated in the control of glucose intake (Hussain et al., 2015), with increased arcuate nucleus GK expression in rats driving increased glucose, but not fructose intake. The authors propose a mechanism whereby glucose drives a positive feedback loop that causes increased NPY release (presumably from arcuate AgRP/NPY neurons) that drives a specific glucose appetite. Although the proposed mechanism of how GK controls glucose intake runs contrary to the classic understanding of how brain GK senses glucose (Ogunnowo-Bada et al., 2014), the study does provide evidence for a role of the arcuate nucleus in the control of glucose intake. Given the limited number of neuronal populations in this nucleus, and the proposed role of NPY release in the response, GK expressed in AgRP/NPY neurons may be a good candidate to drive glucose preference in glucose preference studies.

However, no change in glucose preference was seen in AgRP GK KO mice versus controls, suggesting either AgRP neurons do not control glucose intake, or GK in these neurons does not control glucose intake.

5.5.6 Further Study Plans

Before further phenotyping studies are conducted, it is essential that the success of the AgRP GK KO is confirmed. Using traditional in situ hybridisation probes instead of the newer Exiqon probes, or testing glucokinase antibodies, are options I will consider using. A more quantitative approach, such as qPCR on pools of AgRP neurons, may be better at assessing the knock down efficiency which may vary between mice. This requires crossing an AgRP reporter onto the AgRP GK KO mice, which may take several months.

Further phenotyping studies are planned on AgRP GK KO mice:

- Raise n values for all studies to minimum n=8 per group
- Additional glucose tolerance tests on 18 – 25 week old female AgRP GK KO Mice to confirm phenotype
- TD – NMR to examine body composition, given the differences seen in SF1 neuron GK KO mice (Steinbusch et al., 2016)

Repeat 2 – DG studies without disturbing the mice during the study

6 Chapter 6: General Discussion

6.1 Leucine sensing in the mediobasal hypothalamus

In chapters 2 and 3 of this thesis, three key questions were examined:

- 1) Does leucine, as for fatty acids and glucose, generate neurophysiological responses at the single neuron level?
- 2) What is/are the mechanism(s) by which leucine is sensed in the MBH?
- 3) What is the identity of cells which sense leucine in the MBH?

6.1.1 The neurophysiological response to leucine, and identity of leucine sensing cells

In answer to question 1, in chapters 2 and 3, MBH neurons were demonstrated to be rapidly activated or inhibited by leucine application in adult mouse neuron cultures. Two previous reports demonstrated that leucine increased action potential firing in POMC neurons (Blouet et al., 2009; Smith et al., 2015). Here, I showed that POMC and other MBH neurons respond to leucine with a rapid calcium response, within the physiological range of plasma leucine concentrations after a high protein meal (Blouet et al., 2009).

In answer to question 3, POMC, AgRP/NPY, TH, and other as yet undefined hypothalamic neurons were demonstrated to respond to leucine. Mapping the remaining unidentified leucine sensing neurons onto recently identified hypothalamic populations from single cell sequencing experiments (Campbell et al., 2017) using bioinformatics tools may uncover the identity of these neurons, as visual categorisation using heat maps was not possible. It appears that the leucine sensing population is highly heterogeneous.

6.1.2 The mechanism(s) of MBH leucine sensing

At present, a full answer to question 2 is not possible to give. The calcium imaging combined with the phospho TRAP data suggest the rapid calcium response to leucine may rely on calcium release from intracellular stores. However, the data from experiments with thapsigargin contrast those with ryanodine, 2 – APB, and the single cell sequencing. Experiments using calcium free media suggest the major source of calcium for the leucine response is extracellular. In half of neurons, the rapid calcium response to leucine requires a T Type calcium channel, (first identified in the phospho TRAP study). In all POMC neurons tested, leucine sensing was abolished when this channel was inhibited. Pharmacological studies demonstrated that both the mTORC1 pathway, and leucine metabolism, pathways previously shown to be necessary for mediating some of the effects of MBH leucine on

physiology (Blouet et al., 2008, 2009; Burke et al., 2017; Su et al., 2012), were not involved in the rapid calcium response to leucine. Leucine sensing was also LAT1 independent.

Some studies point to a leucine sensor that resides on the cell membrane, such as a GPCR (Nelson et al., 2002; Wauson et al., 2012). Tas1R1, which dimerises with Tas1R3 in the gustatory system to sense amino acids, was identified as a marker of inhibited neurons in the phospho TRAP data set, but was not expressed in single cells responsive to leucine. Once more cells have been captured and sequenced, the single cell data set can be studied to identify a unique sensor for leucine and/or unique markers expressed only in leucine sensing cells. Validation in calcium imaging experiments and *in vivo* will be required to confirm any findings.

Pathway analysis of the phospho TRAP data set and preliminary experiments (not shown) suggest leucine can regulate actin dynamics. Leucine may be able to modulate the structure of neurons, possibly altering synapse formation. This suggests that leucine activates multiple signalling pathways to coordinate its effects.

Overall, these data suggest that leucine can be sensed by multiple mechanisms within MBH neurons, to mediate multiple downstream effects. Leucine may be sensed by one sensor that controls the activity of multiple signalling pathways, or by multiple sensors unique to each pathway (Figure 6-1). It is not clear from these studies how leucine sensing neurons are organised in the MBH. The multiple effects that leucine mediates on physiology may occur through different subpopulations of MBH leucine sensing neurons.

6.1.3 A model for MBH leucine sensing

The rapid calcium responses observed in chapter 2 were not mediated by mTORC1 and the metabolism of leucine. Previous reports suggest these pathways, and the Erk signalling pathway, can mediate rapid effects of leucine in the MBH and NTS (the contribution of Erk to the rapid calcium response was not tested)(Blouet and Schwartz, 2012; Blouet et al., 2008, 2009). mTORC1 and Erk can control transcription and translation. However, the rapid effects on first meal size and latency, occur within minutes of leucine injection. These responses are too quick to be mediated by changes in transcription/translation, although recent reports suggest transcription and translation can be rapidly activated in synapses (Graber et al., 2013). Preliminary experiments suggest the effects of leucine on actin remodelling in neuron cultures occurred within 30 minutes of leucine application (data not shown).

Therefore, I propose that the rapid calcium response observed in chapter 2 is necessary for controlling the rapid responses to MBH leucine injection on first meal latency and first meal size. The slower responses to leucine, as listed in Figure 6-1, are mediated by mTORC1, Erk and/or leucine metabolism, which control gene transcription/translation and actin remodelling (probably at synapses). These effects, which happen on a longer timescale (minutes to hours) may act to alter neurotransmission: by altering the expression of proteins necessary for propagating action potentials, by altering synaptic strength through changing the structure of synapses, and by changing neurotransmitter gene expression. Whether these sensing pathways exist in the same, or different neurons, is not known.

Future leucine sensing studies should explore the effects of a range of leucine concentrations. Experiments to test the effects of low leucine concentrations on hypothalamic neurons will also be important to perform, given the potential role of the MBH in sensing leucine devoid diets (Maurin et al., 2014), which may be mediated by different pathways to leucine sensing studied in this thesis.

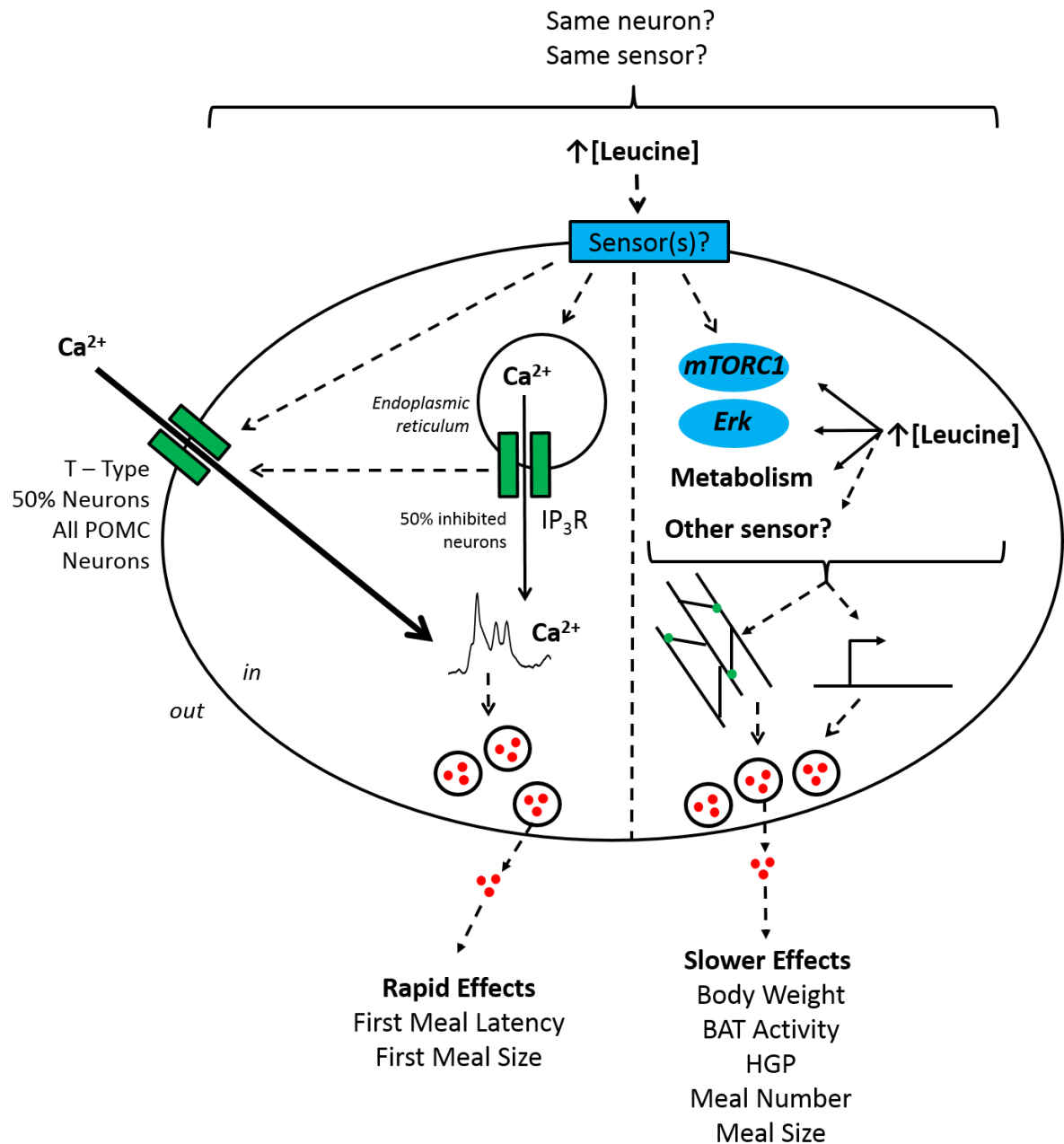


Figure 6-1: Summary of leucine sensing studies, and hypotheses of the downstream effects different leucine sensing pathways control.

Left: the rapid effects of leucine, mediated by intracellular calcium signalling controlling action potential firing and neurotransmitter release. Right: the slower effects of leucine controlled via classical leucine sensing pathways, which may regulate gene expression and actin remodelling. Dashed lines represent unconfirmed links.

6.2 AgRP Neurons and Glucose Sensing

6.2.1 Conclusions and further studies to perform

In chapters 4 and 5 of this thesis, two key questions were examined (with specific hypotheses outlined at the start of these chapters):

- 1) Can the effects of AgRP neurons on glucose homeostasis, as identified by Steculorum et al., 2016, be validated?
- 2) What role do GK expressing AgRP neurons play in the control of physiology?

In answer to question 1, in chapter 4, the results of Steculorum et al., 2016 were not validated. In insulin and glucose tolerance tests, there was no significant difference in blood glucose concentrations in response to AgRP neuron activation. Activation of AgRP neurons after a 2 hour fast caused a reduction, not an increase, in blood glucose concentrations. Steculorum et al., 2016 did not test the effect of activating AgRP neurons on blood glucose concentrations without additional glucose or insulin injected, and so comparing this result to theirs is challenging.

It is surprising that these results could not be validated. One explanation is that the infection of the neurons with the DREADD virus was variable between animals (although this is also likely true in Steculorum et al., 2016). It is possible that in my study either 1) too few neurons were infected to cause a change in blood glucose concentrations, as seen by Steculorum et al., 2016 or 2) a different subpopulation of neurons was infected that do not control blood glucose concentrations. The first explanation requires validation of the number of AgRP neurons expressing the DREADD receptor. In addition to this, the second explanation requires identification of the location and projection of the AgRP neurons expressing the DREADD receptor. As a feeding response of a similar magnitude was observed in my study compared to previous chemogenetic studies (Krashes et al., 2011; Steculorum et al., 2016), it suggests enough AgRP neurons may have been infected. However, the feeding response did vary between animals, and activation of only 800 AgRP neurons has been shown to generate a maximal feeding response, which represents a small percentage of the AgRP neuron population. Therefore, it is possible that I only infected a small number of AgRP neurons, that were able to generate a large feeding response, but a larger number may need to be infected to generate the blood glucose response observed by Steculorum et al., 2016.

The authors did not test how many AgRP neurons had to be activated to generate the blood glucose response (Steculorum et al., 2016).

In answer to question 2, in chapter 5, deletion of glucokinase from AgRP neurons did not result in any change in glucose tolerance, feeding behaviour or glucose preference in the male mice tested. Additional studies and validation of the knock out are required to determine if 18 – 25 week old female mice are glucose intolerant, or if 6 – 10 week old female mice have increased insulin sensitivity.

If the results in female mice are confirmed, why were gender specific effects observed? Others have reported gender specific effects when GK was knocked out from SF1 neurons, with only female mice displaying impaired glucagon secretion and changes in body composition (Steinbusch et al., 2016). However, the reasons for this gender specific effect were unclear. Recent work from the Routh lab (Santiago et al., 2016) demonstrates differences in VMN glucose sensing between male and female mice. This was for two reasons, firstly, there were different proportions of glucose sensing neuron types between genders, and secondly, the responses of some of these groups of neurons varied in the presence of the sex hormone, 17 β -estradiol. This may explain the gender specific effects seen in SF1 neurons (Steinbusch et al., 2016), and similar mechanisms may account for potential differences between the genders in AgRP GK KO mice. Further work will be required to prove this to be true in these glucokinase knock out models.

When additional mice are phenotyped, if it is found that differences between AgRP GK KO females and controls do not exist, the question could be asked what role does glucokinase play in AgRP neurons. Glucokinase may play other roles in these neurons, such as in the control of the counter regulatory response or in the control of body composition as in SF1 neurons. Studies to test these hypotheses will need to be carried out to determine the role that GK plays in AgRP neurons.

6.2.2 Understanding the roles of AgRP Neurons in the control of glucose homeostasis

6.2.2.1 Four subpopulations of AgRP neurons mediating different aspects of glucose homeostasis may exist

Studies suggest AgRP neurons may control four aspects of glucose homeostasis: BAT glucose uptake, skeletal muscle glucose uptake, HGP and glucose effectiveness. These are likely

controlled by different AgRP neuron subpopulations. AgRP neurons have 1 – 1 projections (1 neuron to one downstream site), suggesting one neuron controls one function (Betley et al., 2013). However, one downstream site could control multiple functions. The separate effects reported in different studies (Chhabra et al., 2016; Könner et al., 2007; Smith et al., 2015; Steculum et al., 2016) suggests four different AgRP neuron subpopulations exist, mediating these four aspects of glucose homeostasis.

One outstanding question is why only the effect on BAT was observed when opto- or chemogenetically activating AgRP neurons (Steculum et al., 2016). The proposed effects on blood glucose concentrations when activating the four proposed AgRP neuron subpopulations are outlined in Table 6-1.

Steculum et al., 2016 were the first to report the use of chemogenetics and optogenetics to study the control of glucose homeostasis by AgRP neurons, which is surprising given the first published studies using chemogenetics in AgRP neurons happened 5 years earlier (Krashes et al., 2011). Activation of AgRP neurons (increasing firing) during euglycaemic – hyperinsulinemic clamps did not alter HGP, or insulin stimulated skeletal muscle glucose uptake, but did suppress insulin stimulated BAT glucose uptake, a previously undescribed role for AgRP neurons (Steculum et al., 2016). This role for AgRP neurons in the control of BAT function was recently confirmed by the Blouet lab (Burke et al., 2017). The effect of AgRP neurons on glucose effectiveness was only recently reported (Chhabra et al., 2016), and would not be possible to identify in the studies conducted by Steculum et al., 2016, as an FSIVGTT and modelling must be performed.

Multiple genetic models, as outlined in chapter 4, identified a role for AgRP neurons in the control of HGP, using germline knock out models. Steculum et al., 2016 propose that the absence of an effect on HGP when AgRP neurons are chemogenetically activated is because this effect is due to longer term, MC4R (melanocortin 4 receptor, a GPCR at which AgRP is an antagonist) dependent effects. These effects are only revealed in longer term, not acute, manipulations, such as their previous AgRP neuron specific insulin receptor knock out model (Könner et al., 2007). This explanation requires testing. Opto or chemogenetic activation of AgRP neurons will not be able to examine this effect, unless it can be shown that opto or chemogenetic activation causes AgRP release at synaptic terminals. The MC4R could be deleted in sites at which AgRP neurons project to, and if the ability of AgRP neurons to

increase HGP is disrupted, this would suggest these sites mediate this effect, and that MC4R's are necessary for this process.

6.2.2.2 Understanding which subpopulation GK expressing AgRP neurons are part of
If the effects observed in female AgRP GK KO mice are confirmed with further studies, it is challenging to attribute these findings to a specific AgRP neuron subpopulation without using different experimental techniques. Using methodology such as hyperinsulinemic euglycaemic clamps or the FSIVGTT are required, which provide more reliable measures of insulin sensitivity, HGP and tissue glucose uptake. Performing these technically challenging studies in combination with AgRP chemogenetics, or in the AgRP GK KO mice, may help to understand the functions that AgRP neurons control.

If confirmed, these studies suggest that glucose sensing AgRP neurons control glucose homeostasis, but not feeding behaviour. It would provide further evidence that glucose sensing via glucokinase, a metabolism dependent mechanism, is important in the control of glucose homeostasis.

Table 6-1: Summary of the proposed effects of chemogenetic activation of AgRP neurons on glucose homeostasis

Effect of increasing AgRP neuron activity	↑Muscle Glucose Uptake	↓BAT Glucose Uptake	↑Hepatic Glucose Production	↑Glucose Effectiveness
Blood Glucose Effect	↓	↑	↑	↓
Insulin Sensitivity Effect	↑	↓	-	-
Studies	Smith 2015	Steculorum 2016	Konner 2007	Chhabara 2016

6.3 Nutrient sensing: multiple mechanisms and subpopulations

6.3.1 Novel nutrient sensing mechanisms

Evidence existed before my PhD that nutrients are sensed by multiple mechanisms in the MBH. For leucine, three principal pathways were known to play a role in mediating the

effects of MBH leucine injection. The work in chapter 2 suggests that 1) an additional leucine sensing pathway may exist in the MBH and 2) that two of the pathways previously identified to be involved in the response to MBH leucine are not necessary for this rapid calcium response to leucine.

Phospho TRAP and single cell sequencing were used to profile mRNA expression in leucine sensing cells. However, although a gene may be necessary for leucine sensing, it may not be specifically enriched in leucine sensing cells. Leucine sensing cells may rewire cellular metabolism, possibly through signalling compartmentalisation (Lenne et al., 2006; Nikolaev et al., 2010), or through expressing other genes that themselves are not the sensor, so that leucine sensing by pathways such as mTORC1, expressed in most cells, control unique functions in leucine sensing neurons. This means the candidates identified in the phospho TRAP and single cell sequencing studies will be particularly important to study further, to understand if, and if so, how, known leucine sensing pathways control different functions in leucine sensing cells. What additional genes are expressed to permit leucine sensing?

Further work is required to confirm whether glucokinase knock out from AgRP neurons results in changes in glucose homeostasis in female mice.

6.3.2 Defining nutrient sensing subpopulations

Multiple nutrient sensing populations exist in the MBH. It is likely that leucine sensing neurons overlap with glucose and oleic acid sensing neurons. Single cell RNA sequencing demonstrated that some leucine sensing neurons expressed GK, and other genes involved in glucose sensing, but many of these genes were also expressed in control cells, suggesting the existence of leucine sensing neurons that do not sense glucose. The same conclusions can be drawn about oleic acid sensing neurons, although markers for this population, like amino acid sensing neurons, are less well described. Further studies will be required to understand the overlap between nutrient sensing neurons in the MBH (see section 6.4).

6.3.3 Hypotheses for how nutrient sensing is organised in the MBH

In the introduction, three models were proposed as to how nutrient sensing in the MBH may be organised, with nutrient inputs being organised at the level of the MBH, at the level of downstream circuits, or a mixed model. Work from chapter 2 and 3 has demonstrated that leucine can generate an intracellular calcium response that may alter neurotransmitter release. It is reasonable to assume that amino acid sensing neurons overlap with glucose and

oleic acid sensing neurons (as shown for glucose and oleic acid sensing neurons (Le Foll et al., 2009; Wang et al., 2006)), suggesting integration of nutritional information occurs at the level of the MBH (model A). However, expression of receptors known to be involved in MBH nutrient sensing (which may be necessary for sensing other nutritional information)(Yeo and Heisler, 2012) was low in leucine sensing neurons, suggesting Model B type integration occurs, with hormone sensing MBH neurons projecting with MBH nutrient sensing neurons to downstream targets, where integration occurs.

On the other hand, some MBH leucine sensing neurons express Nav1.7, a sodium channel that allows integration of synaptic inputs over time. This property, demonstrated to occur in AgRP neurons, suggests these leucine sensing neurons could act as integrators of information from other nutrient and hormones sensing sites (Branco et al., 2016), suggesting Model B type integration occurs. However, not all leucine sensing neurons expressed the channel (see chapter 3). For AgRP neurons, known to project one to one (Betley et al., 2013), multiple subpopulations may exist that control glucose homeostasis, supporting Model A type organisation.

Taken together, given that evidence exists for both Models A and B occurring this, supports the existence of a mixed model, Model C (Figure 1-10 in introduction) . The model may vary depending on the function controlled: the CRR to hypoglycaemia may be controlled by the MBH (VMN specifically)(Meek et al., 2016; Sherwin, 2008), but other functions, such as feeding, may be controlled by multiple centres (Sternson and Eiselt, 2017).

Figure 6-3 attempts to summarise the complexity of hypothalamic nutrient sensing. Glucose, fatty acids and amino acids can all be sensed by hypothalamic neurons, and some neurons sense more than one nutrient (Le Foll et al., 2009; Wang et al., 2006). Astrocytes and tanycytes are also nutrient sensing cells, but likely act through neurons (which may not be nutrient sensing) to control physiology (Bingham and Cone, 2015; Perea et al., 2009). Multiple factors listed may alter the nutrient responsiveness of these neurons. Additionally, glucose regulation of the blood brain barrier may alter the access of nutrients to MBH neurons (Langlet et al., 2013).

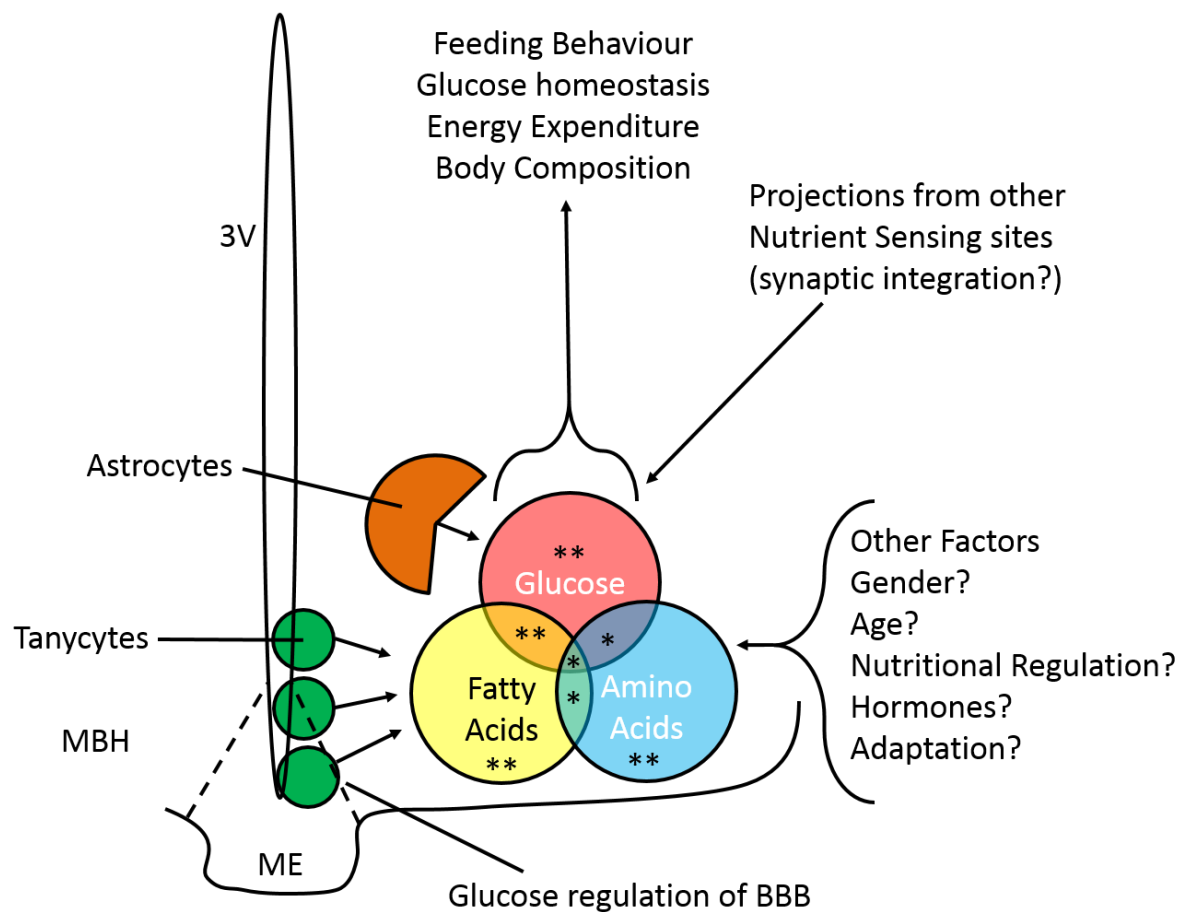


Figure 6-2: Multiple factors regulate the ability of hypothalamic neurons to sense nutrients.

**populations of neurons demonstrated to exist, *hypothesised populations

6.4 Limitations in the current studies and future directions in nutrient sensing research

In the last 10 to 15 years, the repertoire of tools available to neuroscientists has increased substantially, with the arrival of optogenetics, chemogenetics, *in vivo* brain imaging techniques, and single cell RNA sequencing, to name a few (Alexander et al., 2009; DeNardo and Luo, 2017; Harris et al., 2016; Lin and Schnitzer, 2016; Poulin et al., 2016). An elegant review by Scott Sternson and colleagues (Sternson et al., 2016) outlined a framework for deciphering the function of neuronal populations in the control of appetite biology, discussing many of the techniques they have used to study the role of AgRP neurons in the control of feeding behaviour. They detail the range of tools now available, and list the steps that they propose are necessary in a study to fully understand the function of a neuronal population.

The underlying challenge with understanding the function of nutrient sensing neurons is that the nutrient sensing mechanisms are not well understood. Multiple sensing mechanisms exist for each nutrient, but clear evidence for a role of some mechanisms in controlling physiology is lacking. Without knowing: 1) the nutrient sensor, or 2) what allows nutrient sensing mechanisms to control neuronal activity, or 3) a marker for nutrient sensing cells, studies such as those using Cre based technologies are challenging, which are necessary to understand the functions of neural circuits that nutrient sensing neurons project to/control (Sternson et al., 2016). New tools may allow this problem to be circumvented (see later, (DeNardo and Luo, 2017), but these introduce additional challenges. The phospho TRAP and single cell RNA sequencing data sets presented in chapter 3, once completed (for the single cell sequencing), analysed and validated, may identify the leucine sensor(s) in the MBH. However, while studies at the transcriptional level are valuable, protein level studies will be essential going forward to complement these studies.

Proteomics based studies on populations of nutrient sensing neurons may help understand the molecular machinery used by nutrient sensing neurons. Protein level studies are needed because there is not a direct correlation between mRNA abundance and protein expression (Vogel and Marcotte, 2012), with changes in mRNA abundance alone accounting for less than half (depending on the system studied) of the variance observed in protein abundance (Vogel and Marcotte, 2012). This is possibly the biggest drawback of the studies presented in this thesis, and may be the limiting factor in identifying a leucine sensor in MBH neurons.

While single cell proteomics tools are under development, they do not yet have the power to be used for discovery of novel targets (Heath et al., 2016); instead they are used to probe known targets in single cells. However, it may be possible to use proteomics on pools of nutrient sensing hypothalamic neurons of sufficient size (nutrient sensing neurons identified by the range of techniques listed in Figure 6-4). Even these proposed studies have potential limitations, as transcription and translation can occur in sub compartments of neurons (e.g.: at synapses, allowing rapid protein synthesis in response to stimuli, (Richter and Coller, 2015)) that may not be easily collected if the MBH alone is dissected (Graber et al., 2013; Pimentel and Boccaccio, 2014). It might eventually be possible with emerging technologies (or already be possible with techniques I am not aware of) to specifically tag all proteins expressed in an activated neuron (for instance by incorporating a labelled amino acid

specifically into proteins in activated neurons). Then using conventional pull down techniques, or another method, these proteins in dispersed target sites could be identified.

The complexity and number of permutations that could exist in nutrient sensing populations is large. If all the potential combinations of GE/GI, AAE/AAI and FAE/FAI neurons were considered, there could be 26 classes of nutrient sensing neurons, without considering additional modulatory effects of glucose (Le Foll et al., 2009; Venner et al., 2011; Wang et al., 2006; Williams et al., 2008), or the energy status of the cell (Murphy et al., 2009). Using calcium imaging or electrophysiology coupled to single cell RNA sequencing will begin to elucidate the neuronal identity of cells sensing multiple nutrients, which may then help to understand their function. *In vivo* studies such as phospho TRAP, or using emerging techniques where neurons activated by a stimulus can be labelled to allow future manipulation of these cells, may be the most useful tools currently available to begin to understand nutrient sensing. These techniques such as Tet Tagging or CANE, whereby the manipulation performed (typically viral injection of specific constructs) allows stimuli to trigger the expression of Cre (or GFP in some applications) in stimulus activated neurons (reviewed by DeNardo and Luo, 2017). Some of these methods rely on the expression of immediate early genes such as cFOS or Arc, but others use signals such as calcium, to be the signal that triggers Cre expression (DeNardo and Luo, 2017). This allows a much larger range of studies to be performed to understand the function of nutrient sensing neurons (Figure 6-4)(Sternson et al., 2016). Single cell sequencing, as performed by Lam et al., 2017, on these neurons once studies are completed (if a GFP tag could be introduced), would add additional insights.

The application of these tools (Figure 6-4) to nutrient sensing in the coming years will hopefully yield insights into MBH nutrient sensing. These tools will hopefully 1) confirm or identify the mechanisms by which nutrients are sensed, 2) uncover the identity of the neurons which sense nutrients, 3) determine how the MBH coordinates downstream functions in response to nutrient sensing and 4) determine how nutritional information from hormones and other brain regions is integrated to generate a coordinated response from the MBH. This information is necessary to allow more precise manipulations to be performed, and to then understand the consequences of nutrient sensing on physiology.

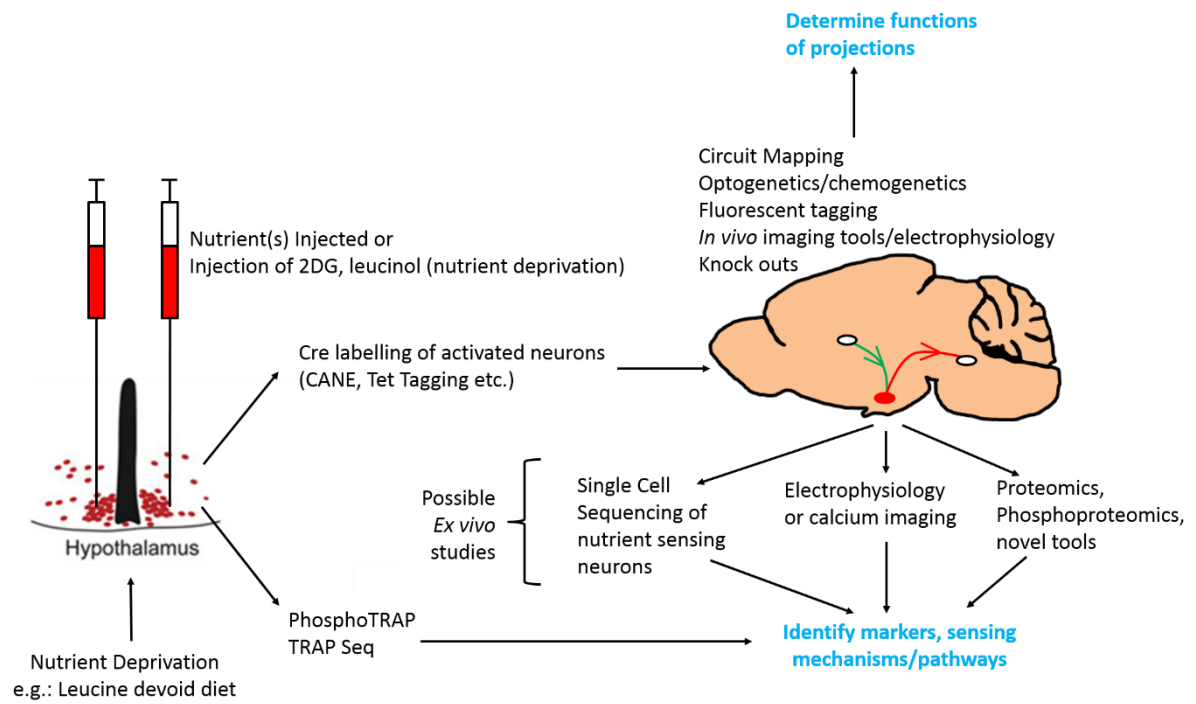


Figure 6-3: The neuroscience toolbox, applied to studying nutrient sensing.

Blue highlights the key aims for these studies. Image of hypothalamus (left) adapted from Knight et al (2012).

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